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The Action of Streptomycin on *Bacterium lactis aerogenes*

BY GWENDA R. BARER

Department of Physical Chemistry, University of Oxford

SUMMARY: The main action of streptomycin on *Bacterium lactis aerogenes* is bactericidal. In an 'untrained' population the number of cells able to survive in streptomycin diminishes with increasing concentration of the antibiotic. If the surviving cells are mutants, many mutant types must be assumed to explain the many degrees of resistance. Ability to survive does not necessarily imply ability to grow in streptomycin. Survivors sometimes have a long lag before growth occurs in liquid or solid media, which suggests that modification of cell protoplasm may be taking place. Colonies picked from high-concentration streptomycin plates often contain so many non-resistant cells that their origin from a single resistant mutant is unlikely. Training to grow in streptomycin may involve a mixed process of selection (by killing) and adaptation. There is close correspondence between the degree of resistance and the training concentration. Partial dependence on streptomycin is acquired. An attempt to 'train' a culture by a method designed to exclude selection was unsuccessful.

Morphological changes were found in untrained cultures grown in streptomycin.

Different samples from one culture frequently showed considerable variation in the number of apparently resistant cells present, depending at least partly on the dilution of the sample and the conditions of spacing on the plate. Resistance is a conditional property; in view of this it is dangerous to construct mutational theories on the evidence of variability between different cultures.

A study was made of the action of streptomycin on *Bact. lactis aerogenes* and of the way in which resistance was developed. It is generally believed that, for those species so far studied (Klein, 1947; Silver & Kempe, 1947; Demerec, 1948), resistance develops when certain resistant cells in the population are selected in the presence of the antibiotic. The resistant cells are believed to arise by mutation before contact between the cells and the antibiotic takes place. The alternative hypothesis, that survival in streptomycin depends on the possession of a variable property, not necessarily due to mutation, and that survivors adapt themselves to growth in streptomycin by direct interaction between cell and antibiotic, has been inadequately explored. Linz (1948*b*), however, considered that his evidence supported the hypothesis of an induced change in the organism. Much evidence for the mutation theory rests on the fact that great variation exists in the resistance of different cultures of the same strain to streptomycin. This evidence may be interpreted in other ways, and the present study has sought to unravel the cause of this variability. An attempt was made to analyse the bacterial population with respect to resistance to streptomycin, before, during, and after single and repeated passages in presence of the antibiotic. It was hoped by this means to show either that every cell became adapted, or that very few cells became selected. The results emphasize the difficulty of the adaptation versus selection problem, and suggest that in some cases both processes may be at work.

METHODS

The organism was *Bact. lactis aerogenes* N.C.T.C. No. 240. This was grown in a simple medium: 10 ml. glucose solution (50 g./l.), or 10 ml. glycerol solution (20 g./l.); 10 ml. KH_2PO_4 solution (9 g./l.), adjusted to pH 7·1; 5 ml. $(NH_4)_2SO_4$ solution (5 g./l.), or 5 ml. asparagine solution (5 g./l.); 1 ml. $MgSO_4$ solution (1 g./l.). The solutions were boiled independently on three successive days and mixed before use (final pH 7·1, for glucose or glycerol). The organism had to be trained to grow in glycerol (Lodge & Hinshelwood, 1944), but the use of this compound avoided the partial inhibition of streptomycin activity caused by glucose (Waksman, 1944). A thermostatically-controlled water-bath at 37° (occasionally 40°) was used for incubating the tubes, which were aerated with a stream of sterile air. Streptomycin was added to media immediately before use. Streptomycin Ca complex (Merck) 1000 units/mg., or streptomycin sulphate (Lilley) 1000 units/mg., were generally used. For plate experiments 2 % agar was added to the above media and the whole autoclaved. Streptomycin was added just before pouring plates (temperature less than 55°). For viable counts the plates were overdried before use by resting them inverted on their lids until they were able to absorb 0·02 ml. drops of medium within 20 min. The method of viable counting was that of Miles & Misra (1938). Tenfold dilutions of the culture in the stock medium were made with pipettes giving standard 0·02 ml. drops (Donald, 1915; Wilson, 1922). Two drops from each dilution were made on each of two plates, and allowed to dry at room temperature. Counts were made daily after incubation at 37°. The advantage of this method is that the count can be estimated from more than one dilution, a fact which proved of great importance. Total cell counts were made with a Hilger's 'Spekker' photoelectric absorptiometer calibrated against a haemocytometer (0·02 mm. depth). For growth curves lasting 3 or 4 days cultures were sometimes packed in ice overnight (Fildes & Whitaker, 1948) with no effect on the curves.

RESULTS

Resistance during first passage in streptomycin

Growth curves. Liquid media containing streptomycin were inoculated (usually with *c.* 6×10^7 cells) from cultures which had just reached the end of the lag phase. Growth curves were constructed from viable counts of samples of the daughter cultures taken at intervals on control and on streptomycin plates (of same concentration as liquid medium). Fig. 1 shows that streptomycin had a bactericidal effect which was delayed in low concentrations; in very low concentrations much growth occurred before killing was evident (Fig. 1*a*). In other instances (Fig. 1*b*) growth and killing occurred simultaneously, since the total count rose while the viable count fell. It is noteworthy that the viable count on the streptomycin plate also fell initially. This may be due to streptomycin being less active under plate conditions than in liquid medium, so that the count on the streptomycin plate at the beginning of the experiment did not represent the number of cells completely resistant to that concentration in liquid medium. Another possible explanation consistent with other observations is that more cells grow on a streptomycin plate than are truly resistant when the inoculum is heavy (e.g. in early samples before killing has occurred to any great extent). The phenomenon might be due to non-resistant cells being able to utilize some substance produced by resistant cells and thus to grow. In later samples the non-resistant cells are dead and only completely resistant cells produce colonies. This explanation fits the fact that the apparent number of resistant cells in a sample diminishes with the dilution of the inoculum, and leads to the conclusion that this number can only be correctly assessed when estimated from a high dilution. When growth eventually occurs the counts on control and on streptomycin plates are usually the same even in high dilutions, suggesting that all cells are resistant. Occasionally after the period of rapid death, there is an interval during which the population is

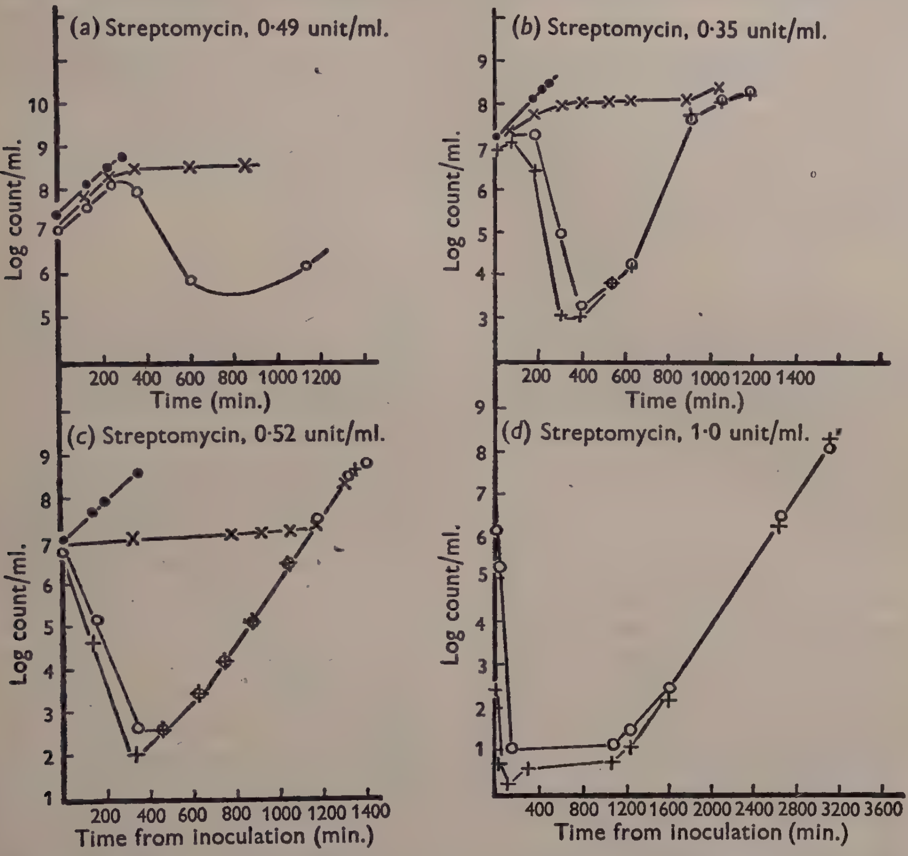

Fig. 1. Growth curves in presence of different concentrations of streptomycin. Glycerol ammonium sulphate medium. 37°. ●—●, Spekker count from control tube; ×—×, Spekker count from streptomycin tube; ○—○, viable count from streptomycin tube on plain agar plate; +—+, viable count from streptomycin tube on plate containing same concentration streptomycin as tube.

stationary (Fig. 1*d*), suggesting that the survivors require some time for adaptation before they can grow in streptomycin. In some cases a single resistant cell in the inoculum could have provided growth much sooner, but it is possible that the interval is due to the fact that the very small number of survivors have a long lag, not due to streptomycin, but similar to the lag pertaining in cultures with very small inocula. The 'small inoculum lag' did not occur with a medium containing asparagine as nitrogen source (Hinshelwood, 1946), and this observation was confirmed for inocula as small as 4 cells with the asparagine medium used in the present work. Nevertheless, long lags (in one case visible growth was not apparent until the eighth day) still occurred in streptomycin when asparagine was used as nitrogen source, so that it remains a possibility that the interval is occupied by adaptation.

Resistance of untrained populations

Population curves. When a culture is inoculated by the viable count technique onto a series of plates containing streptomycin, it is found that the percentage of cells able to survive and produce colonies diminishes with increasing streptomycin concentration. Fig. 2 shows the distribution of the ability to survive in the population. It cannot be proved that the cells which produced colonies on streptomycin plates were resistant initially, since they have grown in the presence of the antibiotic and may have become adapted during the process. However, various facts suggest that there is great variation in resistance in the original population, and that survival is not a matter of chance. On high concentration plates some colonies grow as soon as those on control plates, suggesting complete adaptation from the start. Many colonies appear late, however, and it is possible that adaptation is occurring during this interval. In a given concentration there is much greater variation in the time of appearance of colonies than there is on control plates. Sometimes a second crop appears after the first crop is established, suggesting that the latter provide some substance which the former requires for growth. The possibility that colonies which grow late develop from mutants occurring in minute colonies of non-resistant cells cannot be excluded, especially since such aborted colonies can sometimes be seen by microscopical examination. It is rendered unlikely, however, by the fact that the fully developed colonies may contain a large number of non-resistant cells (see Table 1). In low concentrations (e.g. Fig. 2, 0·5 unit/ml.) where all or nearly all cells survive, colonies appear later than on control plates. A rough picture of the change in population during growth was obtained when viable counts were made at intervals on a series of plates containing different concentrations of streptomycin during the growth of a culture in liquid medium containing streptomycin. It will be seen (Fig. 3*a*) that at first the count decreased on both control and streptomycin plates. When the total count had fallen nearly to the count of 'resistant' cells, growth began. The population curves plotted for different times (Fig. 3*b*), show that when killing ceased, it was mainly the growth of resistant cells that occurred; some non-resistant cells also grew, since the total count remained higher than that on streptomycin, even after growth had recommenced.

Resistance after one passage in streptomycin on solid medium

Relating colonies from higher concentration plates on the same concentration of streptomycin and on control plates gave the results shown in Table 1. The proportion of resistants was usually greater than in the parent culture and

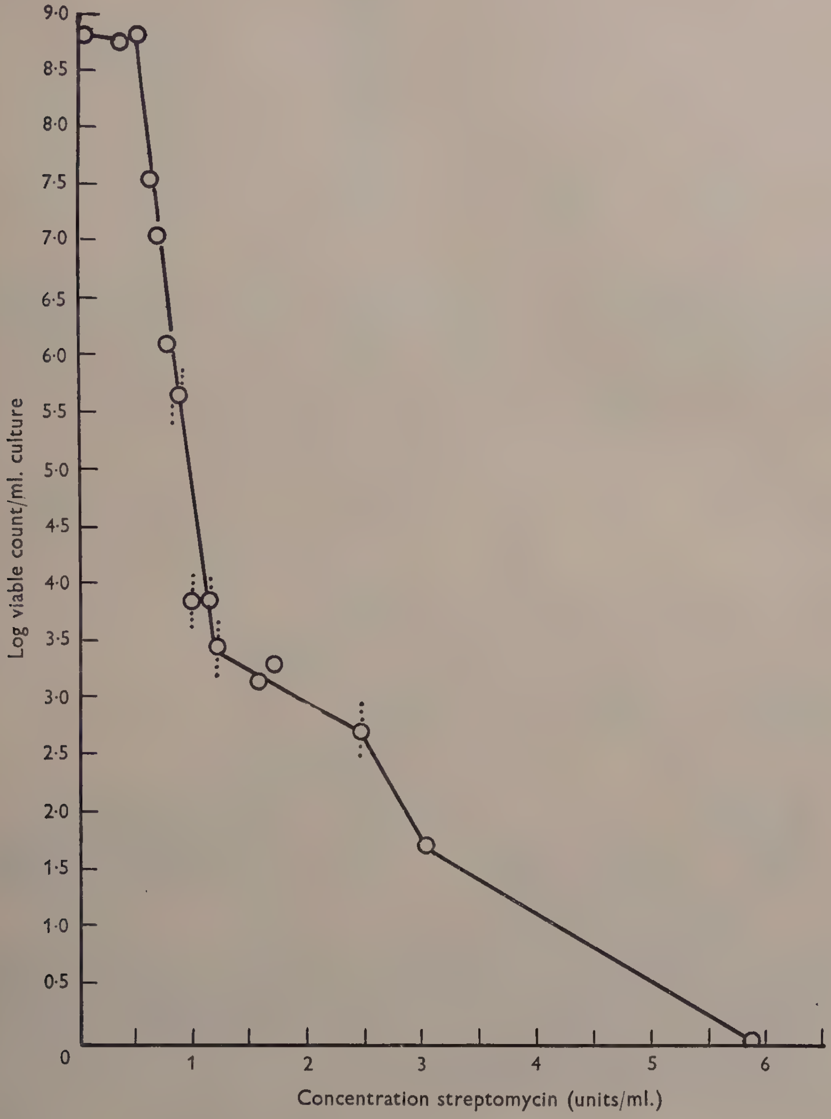

Fig. 2. Typical population curve of *Bact. lactis aerogenes* in streptomycin. Viable counts were made from a culture grown in normal medium at the end of the lag phase of growth on glycerol ammonium sulphate agar plates containing increasing concentrations of streptomycin. Counts were estimated from lowest dilution in which growth occurred. Where exact estimation was impossible (owing to variation between drops, or confluent growth in one dilution and no growth in the next) dotted lines are shown. Points represent final counts after 5 days' incubation.

Table 1. *The resistance of typical colonies from streptomycin plates*

Colony no.	Streptomycin concentration in plate (units/ml.)	Counts on second passage	
		Control plate	Streptomycin plate
		(No. of cells/ml.)	
1	2·5	90×10^6	70×10^6
2	1·3	20×10^6	1×10^6
3	2·3	10×10^6	$0{\cdot}7 \times 10^6$
5	3·3	300×10^6	25
9	3·5	40×10^6	25
11	4·4	7×10^6	*c.* 13
12	4·4	20×10^6	1×10^3
13	8·0	10×10^6	0
14	16·0	2×10^6	0

sometimes equalled the total count (Table 1, no. 1). In other instances the proportion of cells able to grow again on the streptomycin plate was so low (Table 1, nos. 5 and 13) that it seems improbable that colonies could have arisen from single resistant mutants. Such cells could only produce a population of the composition found, for example in Table 1, no. 5, if there were a very rapid back mutation rate combined with a high selective advantage for the non-resistant cells. Those colonies on high concentration plates showed many grossly abnormal cells not seen in controls or in trained cultures, which itself suggests that the colonies did not consist entirely of adapted cells.

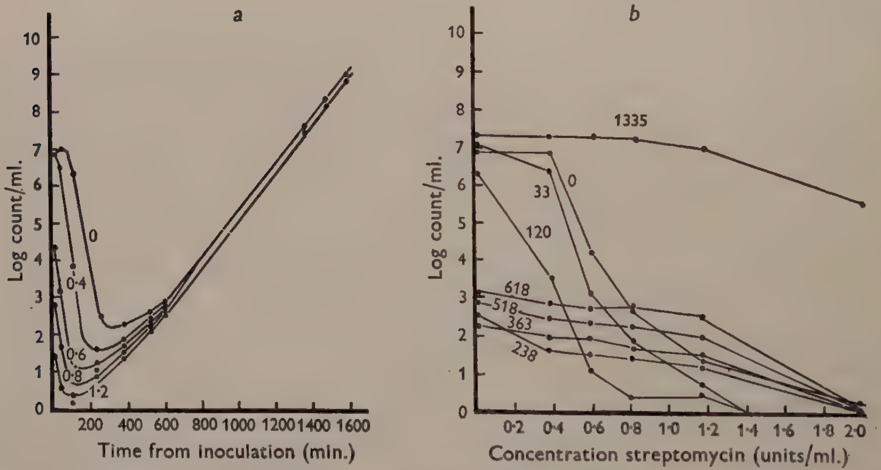

Fig. 3. (*a*) Growth curve of *Bact. lactis aerogenes* in 0·8 unit/ml. streptomycin in glycerol ammonium sulphate medium at 37°. Viable counts made at intervals on control and streptomycin plates of varying concentration. Numbers on curves indicate units streptomycin/ml. (*b*) Population curves during growth of *Bact. lactis aerogenes* in 0·8 unit/ml. streptomycin constructed from same data as (*a*). Numbers on the curves indicate time in minutes from inoculation.

Training to streptomycin on solid medium

An attempt was made to devise a method which would show whether or not every cell in a sample could be made to grow in increasing concentrations of streptomycin, thus excluding the possibility that training was due to selection. Sample colonies from a streptomycin plate on which the full count grew (e.g. with streptomycin 0·5 unit/ml.; Fig. 2) were emulsified, and subjected to viable counts on control plates and on a new series of streptomycin plates. It was hoped that on this second passage the highest concentration on which the full count grew (obtained from control plates) would be increased, and that from this concentration colonies could be picked off and the process repeated. In this way it might be possible to pick off colonies from increasing concentrations, showing that at each stage every cell had grown. This proved impossible for, as in the first transfer, there was either a lower count or delayed appearance of colonies on streptomycin plates compared with control plates (Table 2). Thus the majority of cells could not be trained to grow normally even in low concentrations.

Table 2. *An attempt to train* Bact. lactis aerogenes *to growth in streptomycin on solid medium without selection*

Scheme of training procedure. The inoculations made as under by the viable count technique:

Untrained liquid culture

Control plate — Streptomycin plates
Colony from highest concentration giving count as high or nearly as high as control plate

Control — Control — Streptomycin plates
Again from highest concentration with count as control

Control — Control — Streptomycin plates

And so on — Control — And so on

Resistance of colonies finally tested:

Colony no.	Concentrations of streptomycin through which passed (units/ml.)	Viable counts of colony emulsions (Cells/0·02 ml.): Control plate	Viable counts of colony emulsions (Cells/0·02 ml.): Streptomycin (0·27 units/ml.) plate	
1	0·27, 0·27, 0	1×10^3	0	
2	0·27, 0·27, 0·27, 0·27	4×10^5	2×10^5	Lag* + + +
3	0·27, 0·09, 0·27, 0·18	2×10^3	10	
4	0·27, 0·09, 0·27, 0·27	3×10^6	4×10^6	Lag +
5	0·27, 0·09, 0·27, 0·18	2×10^6	1×10^6	Lag + + +
6	0·27, 0·27, 0·27, 0·27	20	10	
9	0, 0, 0, 0,	2×10^3	1×10^2	Lag +
12	0, 0, 0, 0,	3×10^4	3×10^3	Lag + + +

* Lag = delayed appearance of colonies compared with those on control plates.

Variability in the results of tests

The number of cells capable of giving rise to colonies on a given concentration depended largely on the dilution of the inoculum. Frequently confluent growth was obtained in one dilution, while in a tenfold dilution no colony was found. The opposite phenomenon also occurred (Table 3). Such a breakdown in dilutions did not occur with controls, and appeared to indicate that resistance was a conditional property. The growth of a few cells might provide materials enabling others to grow which would not have grown had they been in an isolated position. Linz (1948*a*) also showed that cells withstand varying concentrations of streptomycin according to their dilution. Much caution must be exercised if counts of resistant cells in a population are to be estimated from a single dilution. In the present work counts were estimated from the highest dilution in which growth occurred; when confluent growth occurred in one dilution and no growth in the next, the count was taken as 1 in the higher dilution. Luria & Delbrück (1943) developed a statistical method for determining the origin of resistance to phage, and other workers (Demerec, 1945, 1948; Ryan, 1948; Scott, 1949) have applied it to other adaptation problems. These workers claim that non-Poisson variation in the counts of resistant cells in different cultures derived from identical non-resistant inocula is strong evidence for the origin of the resistant cells by mutation. Mutation would occur at different times in the different daughter cultures and give rise to clones of varying size. There would appear to be many factors which could cause such variation apart from mutation, the dilution effect being particularly important. However a diluted initial sample (used for inoculation), is sometimes compared, for numbers of resistant cells present, with an undiluted final sample. Experiments of this type have usually been performed on a series of cultures 18–24 hr. old; these would not all be at comparable stages of growth,

Table 3. *The effects of dilution on resistance to streptomycin on solid medium*

Dilution of culture or emulsion	Typical viable counts						
	Control plates		Streptomycin plates (units/ml.)				
			2·4	2·4	3·2	0·4	0·4
	(Cells/0·02 ml.)						
nil	+ + +	+ + +	+ +	+ +	+ +	—	—
1/10	+ + +	+ + +	+	+	2	+ + +	+ + +
$1/10^2$	+ +	+ +	48	52	65	+ + +	+ + +
$1/10^3$	+	+	30	60	23	0	0
$1/10^4$	55	64	11	17	11	0	0
$1/10^5$	7	8	2	0	0	0	0
Source of culture	2·4 units/ml. plate				3·2 units/ml. plate	Untrained liquid culture	

+ + +, confluent growth; + +, nearly confluent growth; +, separate colonies, too many to count.

which is another possible cause of variation. There would also be small differences in count. One culture serves as a control, and from it a large number of samples are taken to determine the sampling variability. These samples are all taken at one time, but if taken at slightly different times they might show a variation comparable to the non-Poisson variation of the whole series. In the present work, it was frequently found that there was great variation in the effect of streptomycin even on identical inocula from one culture; Table 4 illustrates this. Different drops on the same streptomycin plate from a calibrated Pasteur pipette may give very different counts (Table 4, no. 2), and if these drops are spread instead of being allowed to soak in, the count is usually much diminished (Table 4, nos. 3–5). This suggests that material carried over in the inoculum, or the proximity of other cells, may tip the balance in allowing a cell to grow in streptomycin. The counts show a reasonable distribution when the number of colonies is small but when the number is high they are extremely variable (Table 4, no. 5). In this particular medium the maximum population did not remain constant for long, so that no definite variation with age of cells, unaccompanied by a change in count, could be shown. Any trend which may have been present was swamped by differences between samples taken at any one time caused by the factors cited above. These arguments do not exclude that variability is due to mutations, but suggest that there may be other causes which should be taken into account.

Table 4. *Variation in apparent resistance of samples*

(Viable counts were made on streptomycin plates from liquid media at end of log phase.)

Sample no.	Concentration streptomycin (units/ml.)	Counts/individual drops (0·02 ml.) on plate (undiluted)	Counts/5 drops spread across plate
1	3·5 plate 1	3, 6, 7, 7, 3, 5	
	plate 2	0, 6, 1, 0, 2, 5, 6, 11	
2	3·9 plate 1	+++, +++, +++, +++, +++ +++, +++, +++, +++, 50, 50	
	plate 2	+++, ++, 7, 2, 8, 4, 7 13, 4, +, 5	
3	4·4 plate 1	0, 3, 2, 2, 1	0
	plate 2	0, 0, 4, 3, 2	0
4	3·5 plate 1	10, 7	7 (10 drops)
	plate 2	21, 26	70 (10 drops)
5	3·5 plate 1	26, 25, 27, 33, 43	17
	plate 2	+++, +++, +++, 46, 45	76
	plate 3	30, +, +, +, +	34
	plate 4	32, +, +, +, +	50

+++, confluent; ++, confluent with spaces; +, separate but uncountable colonies.

In liquid media variability also occurs. Fig. 4, curve *a*, shows the lag/concentration curve for streptomycin. The time taken to reach a given count is taken as a measure of the effect of streptomycin and plotted against concentration. True lags cannot be used owing to the complex shape of the growth curves, so that a high count, which is only reached in the final growth phase (see Fig. 1), is chosen instead. The effect of a given concentration of streptomycin on a minimum lag culture was extremely variable even under carefully controlled conditions. This was true even when several lag/concentration curves were performed in parallel using similar inocula from the same parent, and appeared to be due mainly to slight shifts in the steep part of the curve. This had profound effects at certain concentrations (e.g. Fig. 5 at concentrations 0·015 and 0·02 unit/ml. streptomycin). If the 'lag', as plotted here, represents the time taken for a few resistant cells to grow, the lag/concentration curve should be capable of being fitted to the population curve. It can be so fitted, but the S-shape of the curve renders this of doubtful value. In general, there is poor correspondence between the effect of streptomycin in liquid and solid media.

Resistance after repeated passages in streptomycin

Fig. 6 shows population curves made at intervals during the training of *Bact. lactis aerogenes* to grow in increasing concentrations of streptomycin. It was usually found possible to increase the concentration by increments of one third after each subculture. When the same process was carried out and at intervals the culture was tested in a series of concentrations in liquid media (in order to plot lag concentration curves) the picture seen in Fig. 4 was obtained. Both experiments showed that there was a close correlation between the average degree of resistance and the training concentration. This could occur on either theory. If there were some enzymic modification in direct response to the antibiotic, the degree of modification could depend on the concentration of streptomycin. If a selection of mutants of varying degrees of resistance were involved, only those mutants able to withstand a given concentration of streptomycin would be selected. Mutants resistant to concentrations higher than the training concentration would not be selected in preference to mutants of resistance equal to the training concentration, but they would nevertheless be selected. Their detection would depend on their frequency. It is difficult to envisage the large number of mutant types closely corresponding to each training concentration which these results would necessitate.

In curve *F*, Fig. 6, more cells grew on a plate containing streptomycin than on the control plate, but at higher concentrations the count decreased. At a further stage of training more cells grew on the highest concentration used than on control plates (Fig. 6, curve *G*). Curve *F* suggests extremely close gearing of the cell's economy to the concentration of streptomycin, such as one would hardly expect from a mutant, which, for example, grew better on streptomycin because it used it as a growth factor. One cannot, of course, show that the actual cells which cannot survive on a control plate but can survive on a 'low' concentration are those which are killed on a higher concentration, though the figures suggest this. The resistant strain was not in the full sense dependent (as were those of Miller & Bohnhoff, 1947, and of Paine & Finland, 1948) on streptomycin. Confluent growth was obtained on control plates in low dilutions, but in dilutions where separate colonies would be expected, none appeared. This is what is found with untrained cultures on streptomycin plates. In liquid media growth was obtained, however, from small inocula of the highly trained strain, in the absence of streptomycin.

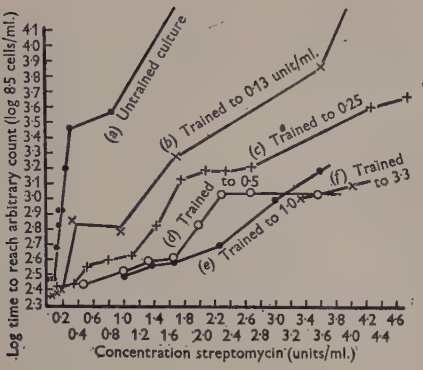

Fig. 4

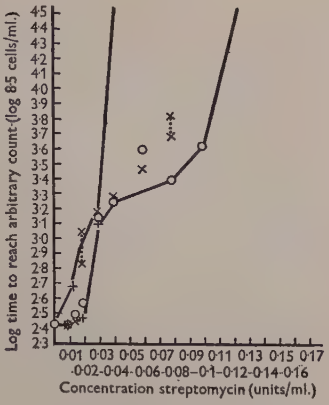

Fig. 5

Fig. 4. Typical lag/concentration curves of *Bact. lactis aerogenes* trained to various concentrations of streptomycin. Glucose ammonium sulphate medium (liquid) at 40°.

Fig. 5. Three lag/concentration curves of *Bact. lactis aerogenes* in streptomycin, to show variability. Identical inocula from same parent.

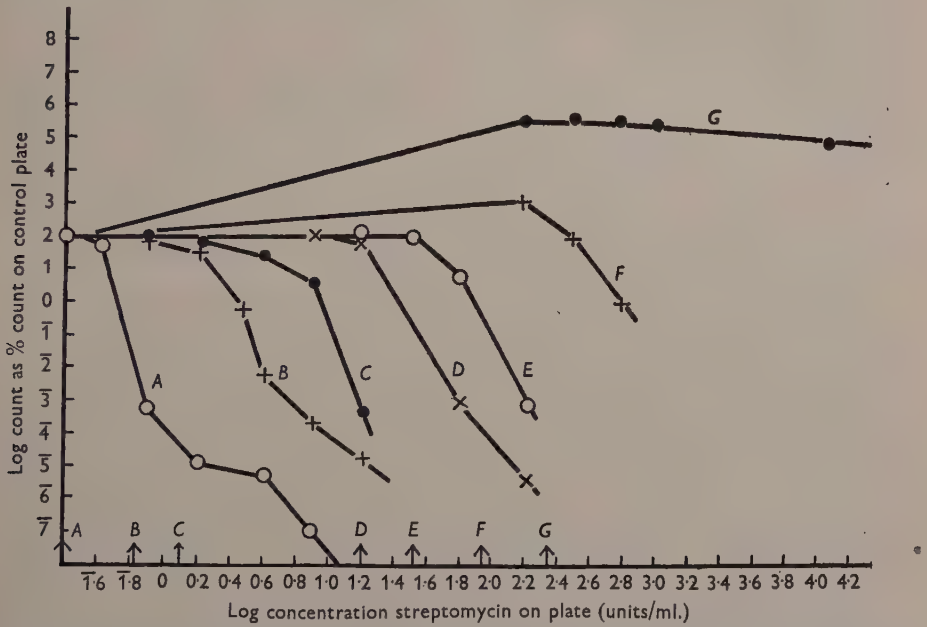

Fig. 6. Population curves during training of *Bact. lactis aerogenes* to streptomycin. Glycerol ammonium sulphate medium containing increasing concentrations of streptomycin. 37°. *A*, *B*, *C*, etc., represent population curves constructed at intervals during training. Arrows show training concentration reached when population curves were constructed.

DISCUSSION

It seems clear that the adaptation of *Bact. lactis aerogenes* to growth in a given concentration of streptomycin involves the selection of those cells able to survive in that concentration. If the surviving cells owe their resistance to mutations which occur spontaneously before contact with streptomycin, the existence of many mutant types must be postulated to explain the many degrees of resistance. Some cells appear, from their rapid growth in streptomycin, to be completely adapted from the start, but most cells grow slowly or after a delay, suggesting the possibility that further adaptation is taking place. An alternative to the mutation hypothesis is that those cells survive which possess an extreme degree of some variable property (this might be a continuous variation depending on a polygenic system, rather than a mutation) and that direct modification of the protoplasm of survivors takes place in the presence of streptomycin. If such modification involved a change in the enzyme balance of the cells which afforded no disadvantage in normal media, it might be copied indefinitely (compare the permanence of antibody formation after removal of the antigenic stimulus). Such enzymic change need not violate the principle of the control of the cytoplasm by genes. The variation could occur within the limits imposed by the hereditary material, and might represent an alteration in the degree of manifestation of certain genes. Manifestation is known to vary with environmental conditions in *Paramoecium* (Sonneborn, 1948). However it must be emphasized that resistance to streptomycin has not been proved to be due wholly to mutation or to adaptation, and although certain of the facts do not readily conform to a mutation theory, none of the arguments are unequivocal. In particular it is difficult to explain the large numbers of non-resistant cells found in colonies on high concentration streptomycin plates; their growth is possibly to be attributed to over-crowding and 'assistance' effects. The fact that the lower the concentration of streptomycin the higher the count reached before killing occurs may suggest that streptomycin, in proportion to its concentration, impedes the availability of some essential substances, and that when these are exhausted death ensues.

The mutation versus adaptation problem in bacteria has become a battleground in biology. It may be worth while to discuss this with reference to the present work. Mutation is usually taken to mean that a new property has arisen spontaneously in the absence of the specific stimulus, but most of the properties studied require the presence of the stimulus for their demonstration. When 'mutants' are detected on test plates (e.g. containing drug, phage, or lacking an essential nutrient) the time of appearance of the colonies is of great importance, since if they are delayed as compared with colonies on control plates, adaptation may be occurring on the plate. This is not always recorded (e.g. Lewis, 1934). Jackson & Hinshelwood (1950), working on the adaptation of *Bact. lactis aerogenes* to growth in D-arabinose, showed that similar counts could be obtained on glucose and on D-arabinose plates, but that the colonies on the latter plates appeared only after a very long lag. The only reasonable interpretation here seems to be that adaptation took place. Even when colonies develop rapidly on test plates they may not be truly resistant since they are growing among large numbers of dead or inactive cells which may assist their growth (cf. Linz, 1948*a*). Also it is instructive to use a series of test plates containing concentrations of the antibiotic or essential metabolite, in order to construct population curves, as the so-called mutants may simply represent the tail of the distribution curve of the ability to synthesize an essential metabolite, or of the property which enables them to resist an antibiotic. This would explain why 'mutants' only appear in cultures which have reached a certain population size. In the present work it was demonstrated that the highest concentration of streptomycin permitting growth increased steadily with population size in a growing culture.

The statistical method developed by Luria & Delbrück has afforded much of the evidence for the mutation theory, but it is open to the objection that all causes of variability other than mutation have not been ruled out. The shape of the lag/concentration curve for streptomycin shows that in a certain range of concentrations small changes in conditions beyond experimental control can cause enormous differences in the result. The resistance of a culture should be checked at several dilutions. In the present work counts of resistant cells were reproducible where the count was low, but extremely variable where it was high. In statistical experiments the diagnosis of non-Poisson variation has often depended on a few high counts. Also the theory of delayed phenotypic expression developed by Newcombe (1948) is based on the fact that two methods of calculating mutation rate on data obtained by the Luria & Delbrück method yield different results. The one giving higher results was the one which included high counts in the calculation, which is just what would be expected if these counts are inflated by 'assistance' effects. Similar objections apply to experiments where large inocula are made into a series of tubes of drug-containing medium. If a small number of these tubes grow, it is no proof that the inocula contained resistant cells, since in high dilution growth might not have occurred, and adaptation might also have taken place in the tube.

In support of an adaptation theory it must be proved that every surviving cell adapts. Methods were devised in the present work in an attempt to satisfy these conditions, but they gave inconclusive results. Much support for this theory is based on its acceptability from the point of view of cell kinetics, the known existence of adaptive enzymes, and the intellectual difficulty of accepting the vast numbers of mutant types which would appear to be necessary to account for strains adapted to grow in innumerable antibiotics, drugs, phages, new sources of carbon and nitrogen, and even a whole spectrum of strains adapted to different concentrations of these agents. This close correspondence of the degree of resistance with the training is particularly clear-cut for proflavine (Davies, Hinshelwood & Pryce, 1944; Davies & Hinshelwood, 1945*a*, *b*; Pryce, Davies & Hinshelwood, 1945) and has been shown in the present work. Other workers, on the other hand, have obtained strains resistant to concentrations higher than that in which they had been trained (Silver & Kempe, 1947; Demerec, 1948; Linz, 1948*b*); although this cannot be completely explained, it seems possible that the discrepancies are due to the use of different methods. Where strains are tested for resistance by a tube method (and this also applies to the egg method used by Silver & Kempe) the highest concentrations in which growth occurs is a measure, not of the *average* resistance of the population (as was measured in the present work) but of the extreme 'tail' of the distribution of resistance in the population, since growth could have occurred from one resistant cell. This would not be expected to bear any relation to the training concentration. Moreover, if the inocula were large, growth of non-resistant cells might have occurred. Linz used fairly small inocula which partially obviates this objection, but on other grounds he interprets his results as due to active adaptation. It is in fact a possibility that strains resistant to higher concentrations than the training concentrations could arise by active adaptation, if the adaptation conferred a selective advantage on the cells.

It is very difficult to design conclusive methods in this field, but that of making viable counts from a liquid culture on to both drug and control plates during a phase of constant population (such as occurs during bacteriostasis due to a drug, or during a lag in a new medium) can give information as to the change in resistance of the population. The number, time of appearance, and rate of growth of colonies on the two sets of plates should be compared. Fig. 7 (*a*) shows the results which might be expected on the two theories for a hypothetical example. It has been claimed that colonies appearing late on a test-plate arise from mutants occurring in microcolonies. In this case some colonies would grow at once followed by a later crop of fairly constant lag; in contrast adapting cells would show a lag which became smaller in successive samples. Repeated population curves made during a phase of stationary population might give results as in Fig. 7 (*b*). If adaptation of the whole population is occurring the full count will grow from the outset on the drug plates (Fig. 7 *a*2, *b*2) because, although adaptation will be incomplete in the early samples it may become complete on the plate. The method of training a culture on solid media by small changes in drug concentration so that every cell could grow (as determined by simultaneous counts on control plates) would provide good evidence for adaptation of the whole population if it could be achieved. The value of the method of replating apparently resistant colonies on further drug plates to see if they are truly resistant has been discussed.

Many results can be explained on either the adaptation or the mutation theory because the tests are inconclusive. Possibly each theory is true at times, and in some cases both may operate. It is reasonable to believe in mutation as the cause of recovery of a synthetic power lost during exposure to X-rays, but not as the cause of origin of a whole series of strains adapted to different concentrations of a drug which the bacterium could not possibly have met before. There is also the possibility that selection may operate not only on mutants but on cells bearing a property showing continuous variation in the population. This might be a more plausible explanation for the origin of many degrees of resistance to a drug, than that of successive mutation steps (Demerec, 1948). There may also be less distinction between the two theories than is apparent, the essential point being whether or not adaptation is related to a specific stimulus. Hinshelwood (1946) pointed out that an increase in enzyme content per cell is a selection of cell protoplasm as distinct from a selection of individuals, but the result on the total bacterial mass is similar. The compromise idea that mutations may be induced by the specific stimulus is only a special case of the adaptation theory—the adaptation of a specific part of the cell. Enzymic expansion might represent the manifestation of a gene which could not show

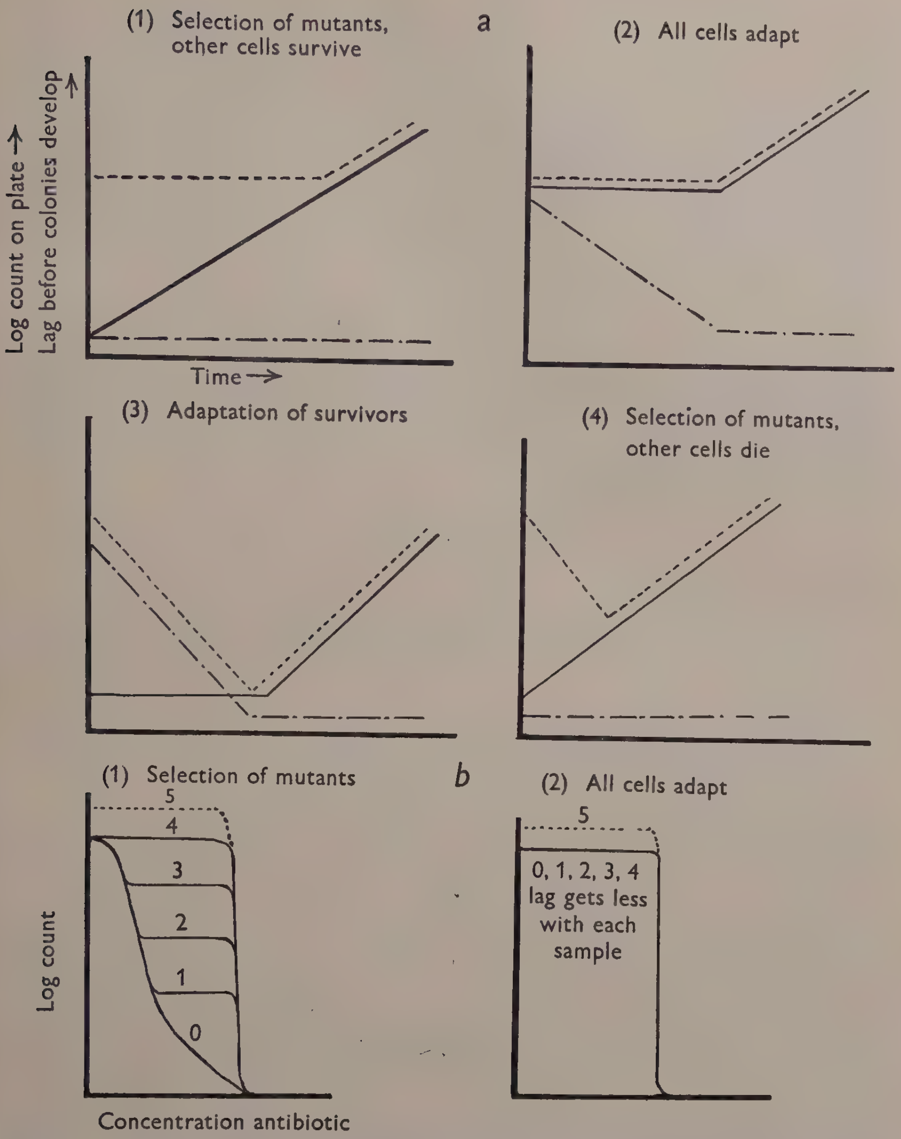

Fig. 7. (*a*) Hypothetical viable counts taken during lag phase of culture adapting to grow in new source of carbon (e.g. *Bact. coli mutabile* in liquid lactose medium). Counts made on glucose and lactose plates. (*b*) Hypothetical population curves constructed from samples taken at intervals from a culture of a bacterium adapting to an antibiotic in liquid medium during phase of constant population (bacteriostasis). Viable counts made on control and antibiotic plates of increasing concentration; in (*b* 1) population not strictly constant as mutants are growing, but since these are few, total population would not appear to alter for some time. (*a*) - - - - - -, count on glucose plate; ——, count on lactose plate; — - — - —, lag before colonies appear on lactose plate. (*b*) Numbers show time sequence of samples, - - - - - shows population curve after growth is apparent.

itself under wild conditions, and this would explain the correspondence between training concentration and resistance. It could provide a meeting ground between the two theories. The conception of the inheritance of acquired characters in bacteria need not be condemned as Lamarckian heresy since in bacteria every cell is a germ cell and subjected to variable conditions from which the germ cells of higher organisms are protected by the constancy of their 'milieu intérieur'. Mutation protagonists seem to attribute all bacterial variation to mutation whereas other processes, such as continuous variation and segregation (where sexual processes occur), have not been excluded. Much more fundamental knowledge of bacterial genetics is required before real advances in this subject can be expected.

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The Photographic Examination of Giant Colonies

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SUMMARY: An apparatus was designed to photograph colonies of micro-organisms on Petri dishes either under conditions of dark-field illumination or of top-lighting. It serves to reveal any motility of the organisms and is valuable as an aid to identification, particularly in the genus *Acetobacter*, members of which have characteristic giant colonies.

Recently (1949) we recorded particulars of giant colonies of *Acetobacter* species which had been grown on a semi-solid medium. In such conditions certain species, e.g., *A. aceti* (Kützing) Beijerinck, form highly characteristic colonies. Other species, including the majority of the known species of *Acetobacter*, characterized by colonies of a particular type, can be classified in a number of groups. Species which form cellulose, e.g. *A. xylinum* (Brown) Holland, *A. kützingianum* (Hansen) Bergey *et al.*, *A. acetigenum* (Henneberg) Bergey *et al.* belong to one group. Again, the giant colonies of *A. acetosum* (Henneberg) Bergey *et al.*, *A. ascendens* (Henneberg) Bergey *et al.*, and *A. rancens* Beijerinck, though differing from each other in size, are similar in general appearance and form another group. A third group comprises *A. suboxydans* Kluyver & de Leeuw, and a number of species all of which are highly motile and form colonies with zones of motile cells. These various characteristic features are brought out particularly well in photographs taken under conditions which we have devised, and since the technique may prove of service to others, we describe it below.

CONDITIONS FOR OBTAINING PHOTOGRAPHS OF PETRI-DISH CULTURES OF MICRO-ORGANISMS

The visibility of small objects depends on their power to reflect, to refract and to scatter light. The smaller the objects the more important is the scattering effect and the less important is reflexion or refraction. The optimum conditions for observing scattering from small objects can be deduced from Rayleigh's equation (1899). Zimm, Stein & Doty (1945), in a recent review, give an expression for the scattering of unpolarized light by objects much smaller than a wavelength. For the present purpose this can be set out as:

$$I \propto (1+\cos^2\theta),$$

where I is the intensity of the scattered light and θ is the angle between the incident beam and the direction of scattering. From this expression it follows that the maxima will be found in the scattered light when $\theta = 0$ and 180°, i.e. when the scattering is backwards or forwards in the direction of incidence. With objects approaching one wavelength of light in size, such as many micro-organisms, there is appreciable interference between the scattered rays in the backward direction, which eliminates the backward maximum. There is then only one maximum in the scattered light, that in the forward direction, which is therefore the best direction in which to look for scattered light indicative of the presence of microscopic objects. In the precise direction of incidence there is much unscattered light from the direct beam which will outshine the scattered light, so the path of the direct beam must be avoided, and the best direction in which to look will be just to one side of the direct beam.

Reflexion and refraction can be considered together, because they are both aspects of the phenomena resulting from the difference of the refractive indices of the object and its environment. When this difference is small, as it is with micro-organisms suspended in solid media, it can be shown that the greatest intensity of both refraction and reflexion from random objects lies near to the path of the direct beam. This direction is therefore the best in which to look for reflected and refracted light indicative of the presence of microscopic objects.

METHOD OF EXAMINATION

The above analysis indicates that the micro-organisms under examination should be illuminated in such a way that the eye of the observer (or the observation lens) will just, but only just, escape the direct beam used for illuminating the preparation. These conditions are therefore optimum for maximum visibility if used with a good black background against which the faintly scattered light is most clearly perceived. To get even illumination for photography the light must come from all sides. This condition is best fulfilled with an annular cone of rays symmetrically disposed about the optic axis of the camera. The required conditions were obtained with a simple apparatus shown in Pl. 1, fig. 1, and diagrammatically in Fig. 1. As shown in the diagram (Fig. 1) the apparatus consists of an annular frame *A* which bears a ring of nine 12 V. 24 W. motor-car headlamp bulbs *O*, connected in series and run direct from the 110 V. mains. (Bulbs (24 V.) could be run from 220 to 230 V. mains, or alternatively a transformer could be used to step down the mains-voltage and the lamps could be run in parallel.) A central stop, which provides a black background is obtained with a cylinder *B* of black paper, closed at the bottom. The incident light is further limited by a diaphragm of black paper *C*. Stouter forms of *B* and *C* can be made from tin. The Petri dish containing the culture *D* to be examined is placed on a ring *E* which is clamped to the support which also bears the ring of lamps and the camera. The support is shown in Fig. 1, where the path of the light rays *OL* is indicated. The central stop *B* and the diaphragm *C* prevent any direct rays reaching the camera lens. It will be seen that the light has only to be deviated through a small angle to reach the camera lens. Thus the illuminating and viewing directions are arranged in the best way to detect faint, scattered, reflected and refracted light from the culture.

To show the raised growth of certain of the cultures the lighting was varied from that in Fig. 1. For deviations from plane surfaces, top-lighting is best, but should not be inclined at too great an angle to the surface, or the elevations and depressions become imperceptible. Top-lighting is obtained by raising the ring of lamps above the plane of the Petri dish. To emphasize the elevation of the colonies still further the light from half the ring of lamps is shaded with black paper and the light from the unshaded lamps is slightly diffused with a semicircular strip of matt celluloid. The photographs in Pl. 1, figs. 2 and 3, and Pl. 2, figs. 4–7, were taken with this illumination.

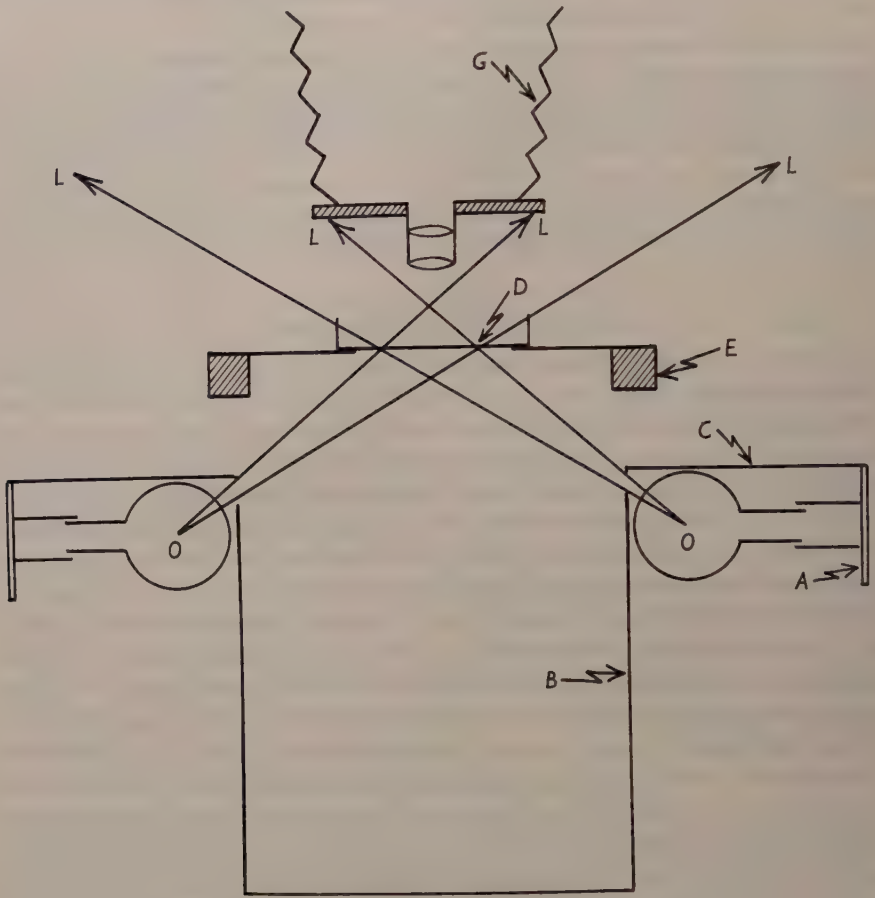

Fig. 1. Diagrammatic cross-section of the apparatus.

RESULTS

Pl. 1, figs. 2 and 3, show a colony of *A. acetosum* photographed with bottom- and top-lighting, respectively. The colony of *A. acetigenum* shown in Pl. 2, figs. 4 and 5, was similarly photographed. Pl. 2, fig. 6, shows a colony of *A. kützingianum*, a species which, in certain media, forms a cellulose surface pellicle. The strong resemblance of this giant colony to that of *A. acetigenum*, which is also a cellulose-producing organism, may be noted. *A. xylinum*, another cellulose former, we found gave giant colonies similar to, though smaller than, those of *A. kützingianum* and *A. acetigenum*. Finally, Pl. 2, fig. 7, shows the large zone of motile cells to be observed in giant colonies of *A. suboxydans*. The strain of *A. suboxydans* used in this work was N.C.T.C.

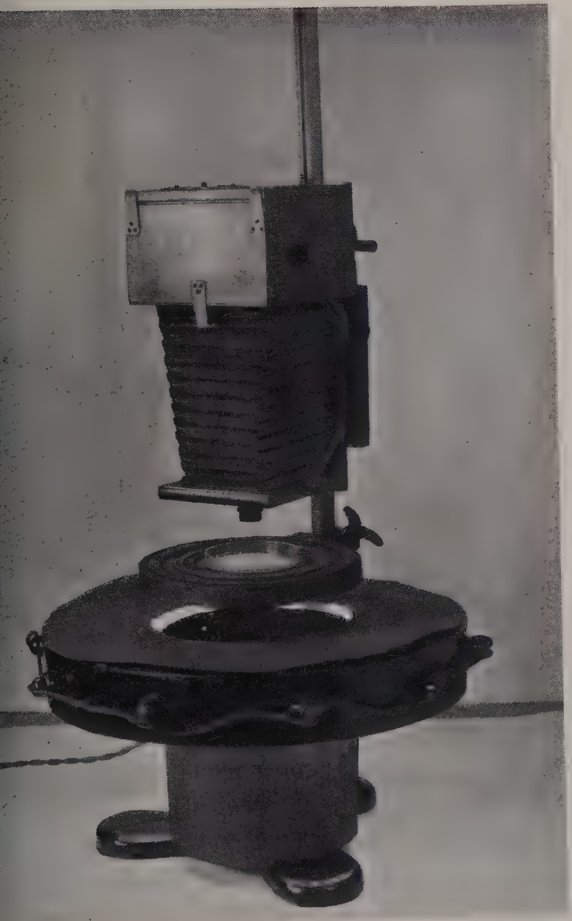

Fig. 1

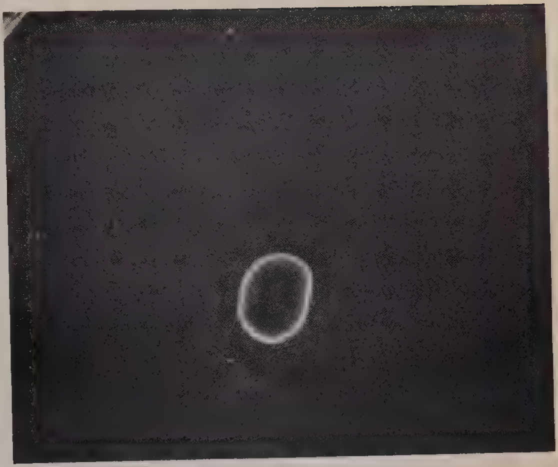

Fig. 2

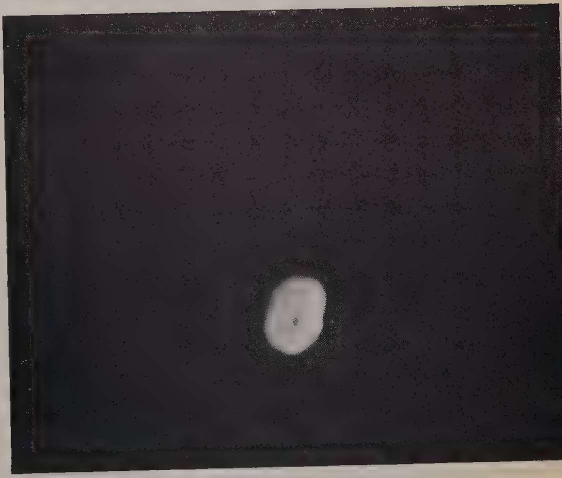

Fig. 3

D. Kulka, J. M. Preston and T. K. Walker—Photography of giant colonies. Plate 1

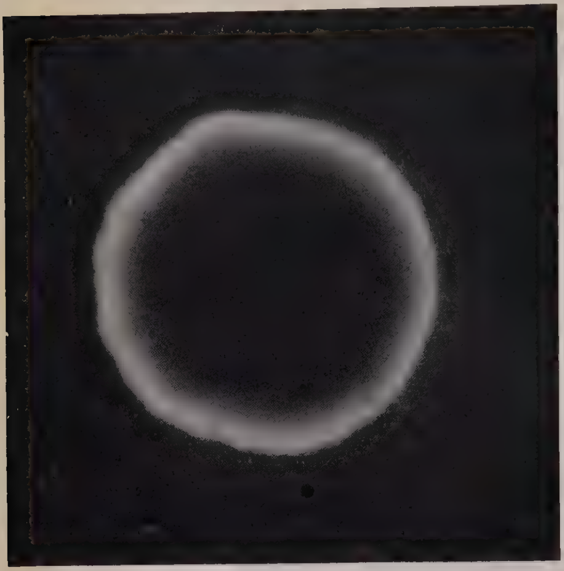

Fig. 4

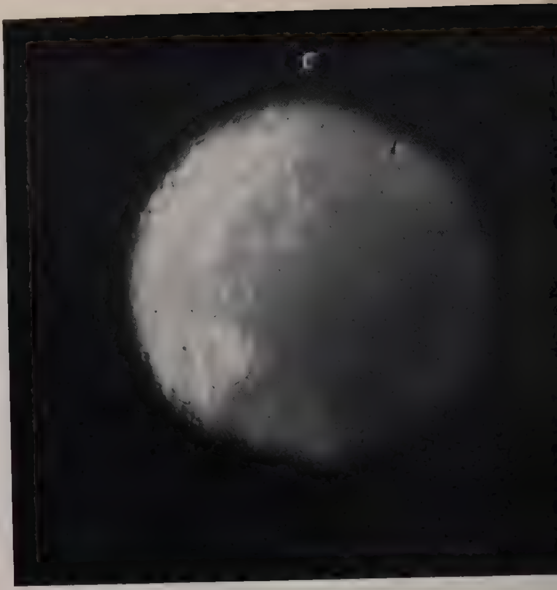

Fig. 5

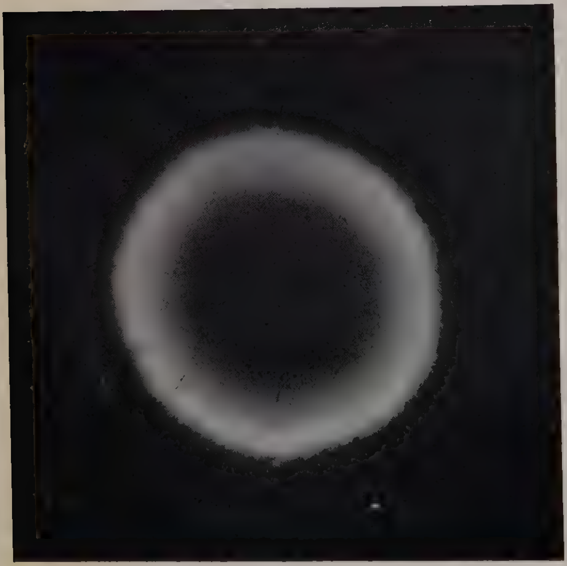

Fig. 6

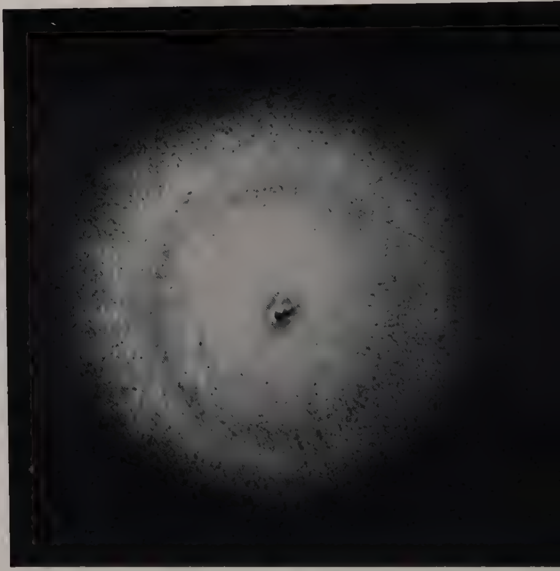

Fig. 7

D. KULKA, J. M. PRESTON AND T. K. WALKER—PHOTOGRAPHY OF GIANT COLONIES. PLATE 2

7069 (A.T.C.C. 621) and was obtained, as were also the other species, from the National Collection of Type Cultures. Other strains of *A. suboxydans* produced giant colonies strongly resembling that shown in Pl. 2, fig. 7.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. General view of the apparatus.

Fig. 2. One colony of *A. acetosum* N.C.T.C. 2224. Dark-field illumination. ×2·9.

Fig. 3. Same colony of *A. acetosum* as in fig. 2. Top-lighting. ×2·9.

PLATE 2

Fig. 4. *A. acetigenum* N.C.T.C. 5346. Dark-field illumination. ×2·9.

Fig. 5. *A. acetigenum* N.C.T.C. 5346. Same colony as fig. 4. Top-lighting. ×2·9.

Fig. 6. *A. kützingianum* N.C.T.C. 3924. Dark-field illumination. ×2·9.

Fig. 7. *A. suboxydans* N.C.T.C. 7069 (A.T.C.C. 621). Dark-field illumination. ×2·9.

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Formation of Clear Zones with 'Sensitive' and 'Resistant' *Staphylococcus aureus* in Penicillin Plate Assays

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SUMMARY: The high-power microscopical examination of stained specimens of portions of agar plate cultures of bacteria allows bacterial cells and colonies to be studied in their original positions. The disposition and morphology of bacteria from the edges of clear zones on penicillin assay plates under conditions sufficiently standardized permitted a distinction between sensitive and resistant strains of *Staphylococcus aureus*. With a sensitive strain 'ghost zones' were formed of partially lysed, distorted and abnormally staining cells; with a resistant strain a 'scatter zone' was formed of apparently normal cells in isolated colonies.

For plate assay work, a sensitive strain may be used when only low concentrations of the drug are available; a resistant strain may be expected to give a sharper zone edge, but will require higher concentrations to provide an adequate response.

Examinations of assay plates have been made by a number of workers in studying the antibacterial properties of penicillin. The wide use of *Staph. aureus* for penicillin assay has prompted the inclusion of penicillin-sensitive strains in such investigations, but a detailed comparative study of assay plates seeded with sensitive and resistant forms does not appear to have been reported.

From direct examination of assay plates, numerous observations on the structure of inhibition zones have been reported. Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey (1941) mentioned that indefinite zones might occur between the clear circle and the opaque normal growth in the cylinder-plate method. Foster & Woodruff (1943) noticed an indistinctness of the clear-zone edge, attributed to lysis of the staphylococci; the variable nature of intermediate zones was also noted. Knox (1945) used a 5 % horse-blood agar medium and a single central cylinder of penicillin solution to each Petri dish, multiple inocula being made in radial streaks. With sensitive staphylococci examination under 10 × magnification confirmed the naked-eye impression that lysis of colonies occurred in the periphery of the cleared part of the streaks. Haemolysis in the region of bacteriolysis suggested that bacterial growth here had previously been vigorous, although only the debris of colonies remained at the end of incubation. With a resistant staphylococcus, lysis was not seen, and the zone of haemolysis did not extend inwards from the region of normal growth.

Vesterdal (1946), using an all-over surface-seeding technique with a sensitive staphylococcus, also noted the extension of clear-zone edges by lysis of marginal colonies, and recorded the diameters of outer and intermediate zone edges. With the lowest concentrations of penicillin used the intermediate zones were narrow and opaque. With greater concentrations these zones widened, and an inner, translucent zone appeared, which gradually displaced the opaque zone, and became still wider as the series was continued to the highest concentrations tested. Vesterdal related the opaque intermediate zone to the development of resistant colonies, made possible by a slower diffusion of penicillin from reservoirs of low concentration, whereas the transparent zone was attributed to lysis. Pulvertaft, Greening & Haynes (1947) found that various combinations of strains and media produced constant patterns of rings with each batch of agar, differing widely between different batches.

Two other lines of study contributed to an understanding of phenomena observed on assay plates: bacterial growth studied in fluid media containing various concentrations of penicillin, and the microscopical observation of organisms while growing in the presence of the drug. The outstanding early fluid culture studies were those of Chain, Duthie & Callow (1945), Kirby (1945*a*, *b*), and Todd (1945) based on nephelometry, and supplemented in the first instance by viable counts and haemocytometer cell counts. Early studies with the incubating microscope were reported by Gardner (1940, 1945), Duguid (1946) and Eriksen (1946). From this work it appears that penicillin-sensitive staphylococci begin to grow normally in low penicillin concentrations, but undergo a gross distortion and lysis when the phase of logarithmic division is reached. It is reported incidentally that the penicillin-resistant type of staphylococcus may not show lysis.

EXPERIMENTAL

In the present work, comparisons were made between the structure of clear-zone edges on assay plates seeded with sensitive and with resistant staphylococci, paying particular attention to penicillinase formation, inoculum size and the occurrence of lysis and morphological change due to penicillin activity.

Method for direct examination of organisms on agar plates

Since direct microscopical examination of the surface of assay plates with a $\frac{1}{12}$ in. oil immersion objective proved unsatisfactory, the following procedure was adopted for examining thin stained slips of agar from the surface of the plate without disturbing the arrangement of the bacteria.

(For the purpose of the following discussion a Petri *plate* consists of a lower, smaller part, the *dish*, and an upper, larger part, the *lid*.)

Flat-bottomed Petri dishes, preferably 15 cm. in diameter, were levelled and poured to a depth of about 5 mm. with ordinary 2% nutrient agar medium containing 5–10% of NaCl to suppress air contaminants (Chapman, 1945). When the medium had solidified, the plate was warmed in the 37° incubator and covered with a layer 0·5–1·0 mm. thick of the same nutrient agar diluted with an equal volume of NaCl broth and seeded with the staphylococcus. A suitable density of seeding is usually attained by adding about 0·1 ml. of an overnight broth culture of the organism to 10 ml. of the diluted agar after melting and allowing it to cool to 40°.

When the second layer of agar had set, the preparation was dried in the incubator for 30 min., with the lid raised. Blotting-paper disks carrying the penicillin solution were then laid on the pre-seeded surface (disks of any diameter between 10 and 2 mm. may be used) and the plate closed. After incubation (not inverted), the lid was raised again and a further period of about an hour allowed in the incubator, to remove free surface moisture. This important step prevented the most superficial portion of the growth from washing to and fro during manipulation.

The plate was then inverted on the bench and 1–2 ml. of Liq. Formaldehyd B.P. (*c*. 39 % (w/v) HCHO) placed in the lid, and the preparation allowed to stand for about 3 hr. It may be left until the following day provided further loss of water is prevented.

Colony dispositions could then be examined directly with the $\frac{2}{3}$ in. objective.

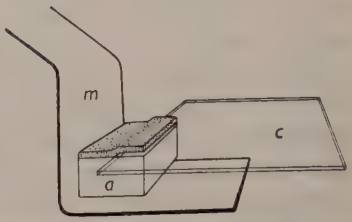

Fig. 1. Technique for separating the agar layers. The full-thickness agar block *a* is steadied in the shoulder of the bent metal strip *m*, while a cover-glass *c* is eased between the foundation and seeded layers.

For examination with high power a rectangular block of agar about 3 × 6 mm. was cut, the surrounding agar pulled away and the block raised without distortion by means of a metal lifter (Fig. 1). With the block steadied against the shoulder of the lifter, a cover-glass was gently eased between the two agar layers and the upper layer removed. If the plate has been sufficiently dried this manœuvre presents little difficulty.

As dehydration with 90 % ethanol at this stage makes the individual colonies slightly more distinct, a few drops are run on to the cover-glass, which is gently rocked to ensure that the agar slip is floating freely in the liquid. This is important as the preparation may be too thick for reagents to diffuse freely through from one side alone.

The slip was washed in water, and floated back on to the cover-glass for staining. Methylene blue stains the cocci a bright sky-blue, and the agar purple. With Gram's method decolorization takes much longer than with a simple smear of bacteria, but contrast is good, as agar is coloured pink less intensely than Gram-negative cells when using diluted carbol-fuchsin as a counter-stain.

The slip may be floated on to a slide, and a cover-glass sealed over it with paraffin wax. If desired, a normal blood film may previously be stained on the cover-glass, and mounted film-side under, to provide an approximate measuring gauge for colonial diameters in different parts of the preparation.

Inoculum density. The most satisfactory preparations were obtained by an inoculum giving an even growth of minute, just-discrete colonies. With penicillinase-producers, the inoculum should achieve this density without diminishing the diameter of the inhibition zone by penicillin destruction (cf. the fluid culture work of Barber, 1947*a*, *b*). The highest tolerated density was determined as follows. When pipette-pen streaks (Ingram, 1951) of graded dilutions of a broth culture were sown at right-angles to a linear penicillin source, at dilutions less than 1/100, the concentration of organisms was inversely related to the distance cleared, but at 1/100 or higher dilutions (to 1/10,000) there was no further increase in cleared distance. The 1/100 dilution of inoculum was therefore used with both the sensitive and the resistant strains, as this dilution satisfactorily fulfilled both criteria.

Organisms used. (1) The 'Oxford' strain, N.C.T.C. No. 6571, supplied in the dry state from original sources by Dr N. G. Heatley, was the sensitive organism. A pipette-pen streak of a 1/100 broth dilution of an overnight broth culture grew on penicillin agar containing 0·001 unit/ml., but not on 0·01 unit/ml. This technique was designed to test cell sensitivity (Luria, 1946). With a very dense inoculum (produced by centrifuging 2–3 ml. of the same broth-culture, and suspending the organisms in 2–3 drops of liquid), a few colonies (most of which appeared to be the minute 'G' forms) grew on the higher concentration, but none on ten times that concentration. Tested by Dr A. W. H. Foxell by Kirby's (1945*b*) method, it produced no penicillinase.

(2) The resistant strain used was one isolated by Martin & Whitehead (1949) from human skin, and was known as 'Lumb C_1'. The biochemical reactions were the same as those of the sensitive strain, except that acid was formed rather more slowly in mannitol and milk. Dr V. D. Allison determined the phage type of the resistant strain to be 6/47 C (weak reaction); he supplied the information that the Oxford 'H' strain did not type with the usual phages, so comparison between the two strains was not possible. A light inoculum produced growth on penicillin agar containing 0·01 but not 0·1 unit/ml., a heavy inoculum growing on 10 but not on 100 units/ml. When growth occurred it was luxuriant and normal; small-colony forms were not observed. Dr Foxell showed that penicillinase was formed by this resistant strain.

Penicillin. Several different batches of penicillin were used; both organisms were exposed to each sample. No differences were detected in the behaviour of either organism to any batch; in particular, lysis of the sensitive organism was similar with each sample (see Steinmann & Nicole, 1945).

RESULTS

The appearances at zone edges formed by the sensitive organism are shown in Pl. 1, figs. 1–3, and by the resistant organism in Pl. 2, figs. 4–6. The thickness of the stripped preparations makes them difficult to photograph and pen-drawings give a better representation of observed phenomena. The only artistic liberty consciously taken has been to represent several focal planes in the same drawing, omitting one set of details in places in order to demonstrate others. Micro-measuring apparatus was not available, but normal human red blood cells were included in the high-magnification drawings to the same scale.

Normal growth

The surface growth of the sensitive organism occurred in small, widely separated patches, and was never more than one cell thick. With the resistant strain surface-growth was free and continuous, and in places several cells thick (cf. Pl. 1, fig. 2 and Pl. 2, fig. 6, upper right-hand corner). The deep growth of the sensitive strain was composed of a mixture of easily distinguished small and large colonies (Pl. 1, fig. 2, C and D), whereas the resistant strain did not show this difference (Pl. 2, fig. 6, lower right-hand corner).

Effects due to penicillin

Penicillin affected both the macroscopic appearances of the zone edge, and the microscopic appearance of the cells and colonies.

Macroscopic appearances of the zone edge

(*a*) With the *sensitive* organism the appearance and structure of the zone edge was the same with all sizes of zone tested, except with certain very narrow zones. Typically, three zones could be distinguished. The innermost zone, A (Pl. 1, fig. 1), was only visible with the hand-lens, and had a sharp and regular boundary with the middle zone B, visible to the naked eye (one of the many types of 'ghost-zone' which may be encountered in assay plates). The third zone, C, had a clear and regular margin at its common boundary with zone B but its outer limit was vague and ill-defined. The B/C boundary is of course that usually measured in assay work.

(*b*) With the *resistant* organism the appearance of the clear-zone edge was entirely different in narrow and wide zones. In narrow zones about 3 mm. wide the edge was indistinct and quite unsuitable for accurate measurement. Many scattered colonies (some much larger than in the normal growth) were found within the clear area (Pl. 2, figs. 4 (i) and 5). No sharp subdivision of the edge into zones was possible, but the areas of scattered colonies were termed 'scatter zones' to distinguish them from the differently formed 'ghost zones' of the sensitive organism. In large zones, 10 mm. or more in width, the edge was sharp and sufficiently well demarcated to be measured to within 0·1 mm. with accuracy, but it was finely and irregularly crenated, so that measurement to this degree of accuracy would not be useful in assay work. The growth in this marginal region was denser than the normal, but was only poorly differentiated from it (Pl. 2, fig. 4 (ii)). With zones of width about 5–8 mm., a transitional form of zone-edge was seen.

Microscopic effects on cell and colony morphology

(*a*) With the *sensitive* organism there was a characteristic cell and colonial morphology in each of the three zones described above (Pl. 1, figs. 2 and 3). In the innermost zone superficial and deep growth was present in about equal amounts, the deep colonies being widely spaced and consisting of few cells only, usually not more than about twenty. The cells were highly pleomorphic and mainly Gram-negative. There were two characteristic abnormalities: (i) groups of two, three or sometimes more enormously swollen and Gram-negative; (ii) cruciate groups of four diplococcal pairs of cells, the inner member of each pair being smaller and sometimes more deeply staining than the outer. Cell debris was found in this region and regarded as evidence of lysis.

In zone B the colonies were much larger, but still smaller and more translucent than normal. There was no differentiation into large and small colonies. Individual cells were mostly Gram-negative and showed many bizarre features. Nearly all the cells occurred in pairs; in many cases the members were unequal in size and sometimes bizarre.

In zone C the colonies were rather larger and more opaque than normal, with a more irregular surface, which may have been due to an increase in the cell diameter, rather than to an increase of the number of cells. Individual cells were uniformly and weakly Gram-positive, and were almost all in pairs of equal, hemispherical members. In this area the plaques of superficial growth were very much larger than normal, but not more than one cell thick.

(*b*) With the *resistant* organism the outstanding and constant feature was the complete absence of any sign of either lysis or of distortion of individual cells. Cells either grew normally in low concentrations of penicillin, or did not grow at all in higher concentrations. Two other observations are also of interest. First, the colonies nearer the penicillin source tended to be larger than normal. In low penicillin concentrations these large colonies were found in the 'scatter zone', but in high concentrations they were confined to a much narrower marginal region just outside the crenated edge of the superficial growth which extended slightly nearer to the penicillin source than did the deep colonies (Pl. 2, figs. 4–6).

Secondly, within the clear area of small and large zones alike there was an even scatter of cells, mostly in ones and twos, normal in appearance and staining reaction, which were thought to be the cells of the original inoculum. This was not found in the preparations of the sensitive strain.

DISCUSSION

A sensitive and a resistant strain of *Staph. aureus* have proved to differ in their reactions on penicillin assay plates. A less elaborate study (Ingram, 1951) of twenty-nine strains suggested that these differences are characteristic of the two kinds of staphylococcus, in agreement with the findings of other workers. If these results are confirmed on a larger scale, it would seem that the sensitive kind shows a higher cell sensitivity to penicillin, does not produce penicillinase, and undergoes distortion and lysis when destroyed by this substance, whereas the resistant kind shows a lower cell sensitivity, produces penicillinase, and does not undergo distortion or lysis. Thus, in choosing a test strain for plate assay work, the following considerations may be applied: a sensitive strain will allow relatively low concentrations of penicillin to be assayed, but will carry the disadvantage of an unpredictable number of 'ghost zones' which may be formed by distortion, lysis and, perhaps, secondary growth (Vesterdal); on the other hand, if higher concentrations of penicillin are available for assay, a resistant organism will probably give sharply defined but crenated zone edges with zones above a certain diameter. Below that diameter a 'scatter zone' will be present, formed presumably from more-resistant variants present in the original culture. Comparisons (Ingram, Foxell & Armitage, unpublished) of the dose-response curves obtained in plate assays made with the sensitive and resistant strains used here, have shown the form of the curves to be similar, despite the production of penicillinase by one strain.

One feature common to the zone edges of both strains was the dense region outside the clear area, fading insensibly into normal growth. 'Secondary zones of stimulation' seen on assay plates with the standard American assay strain of *Staph. aureus*, FDA 209, were referred to by Cholden (1944), perhaps with this effect in mind. Mitchison & Spicer (1949) observed that colonies were equally numerous in the similar dense zone in a streptomycin assay, and believed that the increased density could not be explained by 'considerations of living space' alone. Dufrenoy and Pratt (Pratt & Dufrenoy, 1947; Dufrenoy & Pratt, 1947, 1948) described this dense area explicitly, and after a number of physical and chemical investigations showing cell metabolism to be normal in this region, concluded that the denser growth was due to a stimulation by subtoxic concentrations of penicillin. This interpretation is perhaps also supported by the evidence of Miller, Green & Kitchen (1945) that staphylococcal populations may increase to higher densities in the presence of subtoxic penicillin concentrations than in control cultures. Such an hypothesis of 'drug stimulation' requires further confirmation, and the dense zone may be more simply explained as the result of the marginal position of the cells concerned. Here they may derive nutriment from a wider field than is available to cells entirely surrounded by others. At the margins of any continuous expanse of bacterial growth dense zones may be seen, formed no doubt in this way, for Mayr-Harting (1947) has shown that, within wide limits, the number of bacterial divisions that can occur on a given area of nutrient surface depends upon available pabulum, and is independent of the size and proximity of individual colonies. Without information on cell distributions the argument of Mitchison & Spicer is unconvincing.

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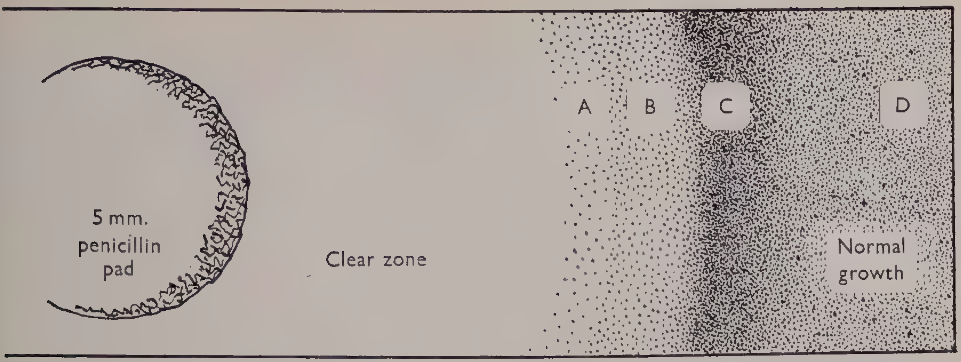

Fig. 1

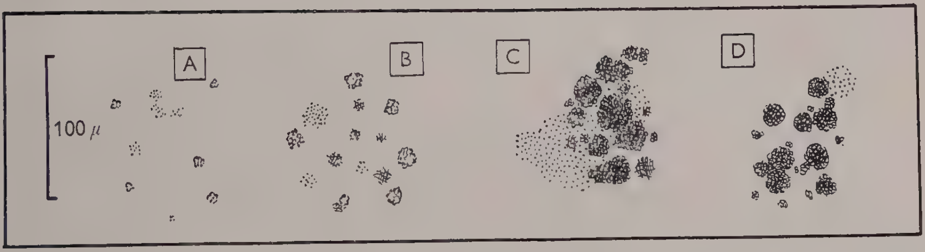

Fig. 2

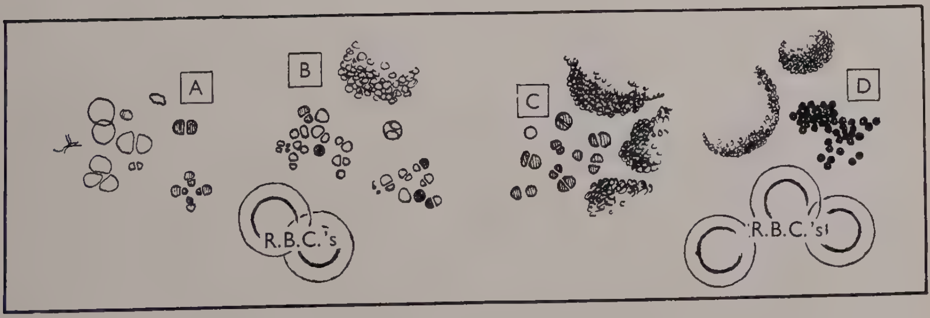

Fig. 3

I. C. Ingram—Zones on penicillin assay plates. Plate 1

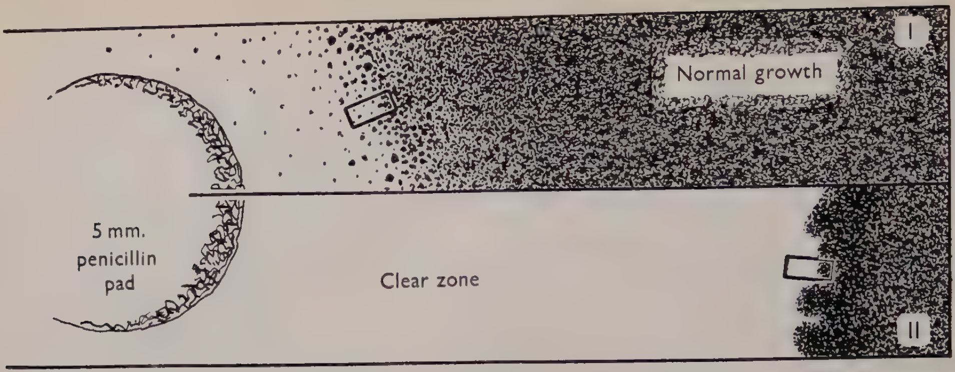

Fig. 4

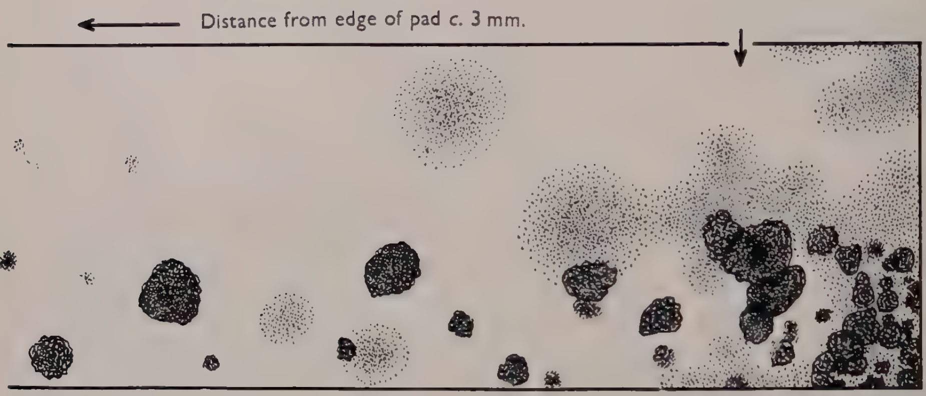
Distance from edge of pad c. 3 mm.

Fig. 5

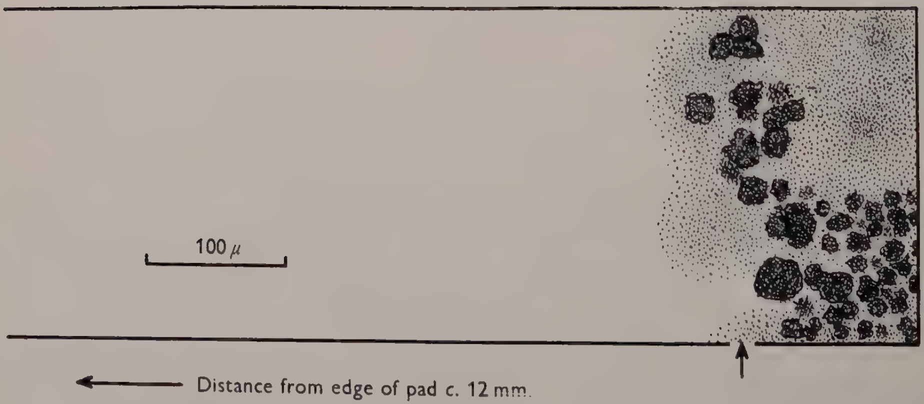
100 μ
Distance from edge of pad c. 12 mm.

Fig. 6

G. I. C. Ingram—Zones on penicillin assay plates. Plate 2

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EXPLANATION OF PLATES

Plate 1

Figs. 1–3. The sensitive strain of *Staph. aureus*.

Fig. 1. General appearances at the zone-edge formed round a disk reservoir on a surface pre-seeded plate. Lettered areas shown enlarged below. Unstained, fresh preparation: hand-lens examination.

Fig. 2. Colonial appearances in the lettered areas of fig. 1. Relative extent of plaques of superficial growth shown by stippling; deep colonies drawn dark. Unstained, fresh preparation: $\frac{2}{3}$ in. objective.

Fig. 3. Cell morphology of the same areas. Colony profiles intended only to show general intensity of staining. Gram-stained preparation (technique described); $\frac{1}{12}$ in. oil immersion. Red blood cells included for scale.

Plate 2

Figs. 4–6. The resistant strain of *Staph. aureus*.

Fig. 4. General appearances at the zone-edge formed on a surface pre-seeded plate round disk reservoirs carrying low (i) and high (ii) penicillin concentrations. Enclosed areas shown enlarged below. Unstained, fresh preparations, comparable with fig. 1. Hand-lens examination.

Fig. 5. Colonial appearances in the area enclosed in fig. 4 (i). Deep colonies (drawn dark) omitted from the upper half of the drawing to show superficial growth alone (stippled). Unstained, fresh preparation, comparable with fig. 2: $\frac{2}{3}$ in. objective.

Fig. 6. Colonial appearances in the area enclosed in fig. 4 (ii). Deep colonies omitted from the top right-hand part of the picture to show how the superficial growth was continuous and in places heaped up. Technique and scale as in fig. 5.

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Classification of Staphylococci by Penicillin Lysis

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SUMMARY: In twenty-nine of a series of thirty strains of *Staphylococcus aureus* and *albus* there was correspondence between penicillinase production, 'cell sensitivity' to penicillin and penicillin lysis, but no relation between these characteristics and the results of eleven routine tests for pigmentation, β-haemolysis, coagulase formation and various biochemical reactions.

It has been recognized for some time that staphylococci may be divided into those which do and those which do not produce a penicillinase (Hobby & Dawson, 1944; Spink, Ferris & Vivino, 1944; Kirby, 1945*a*, *b*). Also, staphylococci are often tested for their penicillin sensitivity (tolerance) and classified as 'sensitive' or 'insensitive'. The literature contains a number of references to another staphylococcal characteristic *vis-à-vis* penicillin which might be used as a third criterion of classification, namely penicillin lysis.

The phenomena of autolysis and lysis by drugs are classical in bacteriology; the subject has recently been reviewed by Pulvertaft & Lumb (1948). Penicillin lysis of staphylococci was observed by Fleming (1929), Smith & Hay (1942) and Gardner (1945), and related to turbidity measurements by Chain, Duthie & Callow (1945) and Kirby (1945*a*). Knox (1945) and Todd (1945) related the occurrence of lysis to the growth-phase of the organisms, as did Chain *et al.* by making cell-counts and viable counts of their cultures. A comparison between the chemical activity of areas of lysis and areas of growth was made on assay plates by Pratt & Dufrenoy (1947), who showed that lipids were liberated in areas of lysis, and hydrolysed into fatty acids. The resistance of certain staphylococcal strains to lysis was noted by Kirby and by Knox, being associated with relative tolerance of the bactericidal action of penicillin. Kirby (1945*b*) also demonstrated the presence of penicillinase in unlysable, resistant strains. In view of the reports of Kirby and of Knox, a study was made of the correspondence between penicillin lysis, penicillinase production and penicillin 'cell sensitivity' (Luria, 1946) of a small number of strains of staphylococci, which were also tested for other characteristics.

METHODS

Thirty-seven strains of staphylococci were investigated. They were mostly obtained from routine clinical material, and were stored at room temperature on nutrient agar slopes, and tested in batches. Seven strains are reported separately, as they produced so much penicillinase that no lysis was observable with the penicillin concentration employed in the test. The main investigation is therefore concerned with thirty strains.

Technique of plate inoculations

Pyrex Petri dishes, 15 cm. diameter, were used. Particular attention was paid to standardizing the inocula on all the plate cultures; for seeding, the following modification was evolved of Heatley's (1947) Pasteur pipette pen. Standard thin-wall glass capillary tubing of 1·5 mm. external diameter was obtained in 4 in. lengths, the terminal centimetre of each bent to make an angle of about 100°, and the cut edges of the tip of the bent portion smoothed without decrease in lumen. A pivoted holder was devised for seeding from the capillaries under standard conditions which deposited a repeatable, even inoculum (Fig. 1).

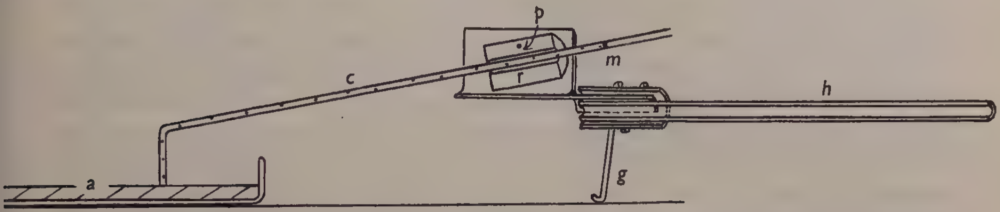

Fig. 1. Capillary pipette pen. The bent capillary *c* is gripped by the section of thick-walled rubber tubing *r* which is pivoted at *p*. The capillary is filled to the mark *m*, and the tip rests vertically under its own weight on the surface of the agar *a*. By means of the holder *h* the tip of the capillary is drawn over the agar at right-angles to the plane of the diagram, with the guides *g* (only one of the pair is shown) resting on the bench. Provided the height *ap* is constant, the angle of *h* is immaterial.

The estimations of cell sensitivity were made with *light* and *heavy streaks*. The *light streaks* were 1/100 to 1/1000 dilutions of 24 hr. broth cultures, and the *heavy streaks* the centrifuged deposit from similar cultures suspended in a few drops of broth (Ingram, 1951).

Bacteriological tests

The following investigations were made on all strains:

Plate cultures (*heavy streaks*). β-Haemolysis and pigment production on human blood agar (0·5 % NaCl); coagulase and pigment production on 20 % human plasma agar (Reid & Jackson, 1945). These plates were incubated at 37° and read after *c*. 24 hr.

Liquid cultures. Coagulase production by a 6 hr. tube method used in this laboratory as a routine; production of acid and clot in Hiss's serum water containing glucose, sucrose, lactose or mannitol; and production of acid and curd in litmus milk. These were incubated at 37° and read daily for a week. Strains were classified as coagulase positive when either the plate or the tube test was positive. Similarly, classification was by the darker pigment produced on either blood agar or plasma agar.

Stab culture. Liquefaction of gelatin at room temperature, read daily for a week.

Penicillin tests

The following investigations were made:

Penicillin-dilution plates. Following the techniques of Foster & Woodruff (1943) and Frisk (1945), a series of penicillin dilutions were made in molten agar, and poured in Petri dishes. When set, surface light streaks were made with the modified Heatley pen. After incubation the results were recorded by noting the highest concentration of penicillin that permitted growth, and the lowest that inhibited growth, in the series of plates that was used, for example, '0·01–0·02'. This value was taken as the cell sensitivity, but was not determined for all the strains because the technique was unduly time-consuming.

Penicillin-diffusion plates. Suggested by the technique of Mohs (1945), and following Fleming's (1929) and Barber's (1947*a*) ditch-plate principle, heavy and light streaks of each strain were sown with the pen in parallel lines on the surface of plain agar plates, each also carrying three streaks of the Oxford staphylococcus 'H' (N.C.T.C. 6571). Across each plate at right-angles to the streaks was then laid a strip of thick blotting-paper evenly saturated with penicillin solution of 10,000 units/ml. After incubation the results were read as follows: measurement was made of the distance from the edges of the penicillin reservoir to the limits of growth in each streak, and the distances obtained with the test organisms were expressed as a percentage of the mean distance in the three control streaks on the same plate. This value is referred to as the *percentage inhibited distance.* The measure does not imply that a linear relationship exists between penicillin concentration and 'inhibited distance' (this relation is usually taken to be logarithmic), but the convention is simple and sufficient to establish large differences between groups. The percentage inhibited distances of the light streaks were regarded as an approximate indication of the 'cell sensitivity' of those strains in which this value was not determined by the plate-dilution method.

The heavy streaks on the same preparations were used for the observation of lysis, which when present was found at the junction of free and inhibited growth (cf. Fleming, 1929; Knox, 1945).

Method of soaking linear penicillin reservoirs

Thick blotting-paper is cut into suitable strips of even width which are saturated by immersion in penicillin solution, and the excess run off. They are drained by laying on a firm pad of absorbent material and gently pressed into contact. When it is seen that excess of moisture has been taken up, usually in 2 or 3 sec., the strips are transferred to the assay plate. An ordinary asbestos cooking mat has been found satisfactory for the absorbent pad, and it can be sterilized in the flame from time to time. The method was shown to produce an even saturation of individual strips and a reproducible impregnation from strip to strip by statistical analysis of experiments with multiple streaks. Fig. 2 shows the clear zones produced on surface pre-seeded plates by strips soaked in various penicillin solutions, and Table 1 gives the analysis of variance for the test of strip to strip reproducibility.

Table 1. *Analysis of variance for test of strip to strip variability*

Contribution	Degrees of freedom	Sum of squares	Mean square (variance)	Variance ratio
Between doses: Regression	1	476·58	476·58	1604
About regression	6	3·81	0·635	2·14
Doses totals	7	480·39		
Within doses	40	11·89	0·297	1·00
Totals	47	492·28		

The $P=0\cdot05$ limit for $n_1=6$, $n_2=40$ is 2·34, so deviations from regression are not significant. The variance ration for linear regression is clearly very significant.

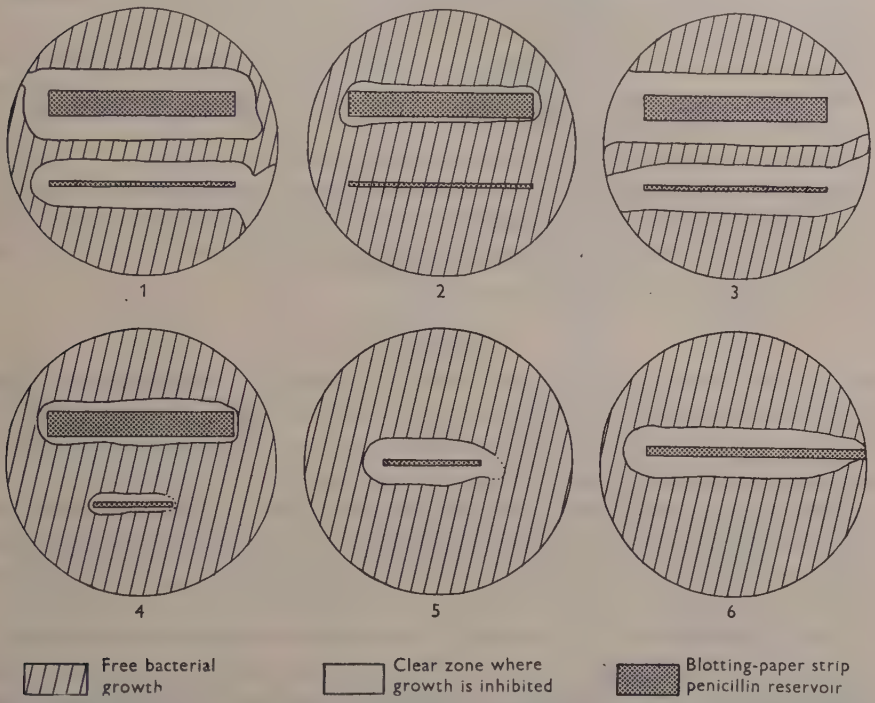
1
2
3
4
5
6
Free bacterial growth
Clear zone where growth is inhibited
Blotting-paper strip penicillin reservoir

Fig. 2. Tracings of the outlines of growth after incubating evenly-seeded agar plates (*Staph. aureus*) carrying blotting-paper strips soaked in penicillin solutions. The broken outlines indicate an indistinct margin between areas of free and inhibited growth.

Plate 1. Two strips soaked and drained by the described technique, carrying 10,000 units/ml. Wide clear zones were formed, regular over a long central portion, and of the same width with both strips. Irregularities were found at the ends of the strips, with the solution 'creeping' to the edge of the plate.

Plate 2. Two strips similarly soaked, carrying 100 units/ml. An uneven, narrow zone was formed round the wide strip, and no zone round the narrow strip.

Plates 3 and 4. Two strips each similar in size and penicillin concentration to those on plates 1 and 2, soaked but not drained on the absorbent pad: excess solution was merely run off before the strips were laid on the plates. Plate 3 gave similar appearances to plate 1, except that there was more 'creeping'. Plate 4 showed a substantially similar result to plate 2, the difference in zone width being presumably due to the greater absolute quantity of penicillin carried over in the reservoirs.

Plates 5 and 6. One end only of each strip soaked in penicillin solution 10,000 units/ml., and not drained. 'Creeping' had occurred in both strips, so that some clear zone was seen along the whole length of the strips. Gross irregularities were obvious.

Test of strip-to-strip reproducibility: analysis of variance

Eight strips were soaked by the described technique in a twofold dilution series of penicillin solutions containing 10,000, 5000, 2500, ..., 78 units/ml. and laid at right angles over parallel streaks of a penicillin-sensitive *Staphylococcus aureus* (N.C.T.C. 6571) on a 27 × 27 cm. agar slab, so that each strip crossed six streaks. The paired responses obtained from each crossing were averaged. The above analysis (Table 1) of the forty-eight means shows that the impregnation was sufficiently well reproduced from strip to strip for the deviations of strip means from the general regression to be less than the scatter of the readings within individual strips. The correlation coefficient, r, was +0·9960. The actual deviations of the strip means from the regression line were: +0·08, −0·12, +0·06, −0·36, +0·25, +0·53, −0·10 and −0·36 mm. The standard deviations of individual strip means lay in the range 0·831–0·386 cm., mean 0·578 cm. (coefficients of variation 3·34–1·42 %, mean 2·47 %).

Penicillinase tests. Twenty-six strains were tested qualitatively for the production of penicillinase, either by Kirby's (1945*b*) adaptation of Harper's (1943) extraction, or by a 'hole-plate' or 'pad-plate' technique similar in principle to that used by Barber (1947*a*), or by a 'parallel-streak' method similar to that of W. Hayes (unpublished).

RESULTS

Twenty-nine strains were classified without difficulty by the criterion of lysis; a thirtieth strain could not be classified. Fig. 3 shows the characteristic appearances of the two types of strain when growing at right-angles to a linear penicillin source. Nineteen strains were lysed and ten were not.

Bacteriological tests. The differences between strains in the bacteriological tests were entirely uncorrelated with lysis; in particular, yellow and white pigmented strains and coagulase positive and negative strains occurred in both groups.

Special penicillin tests. By contrast, the penicillin tests showed a consistent distinction between the two groups. The nineteen *lysed strains* were all relatively sensitive; the percentage inhibited distances of the light inocula lay within the range 88–112 %, that is to say, these organisms all had about the 'sensitivity' of the Oxford strain. In eight strains of this group the cell sensitivity was accurately determined by the plate-dilution test, and lay within the range 0·01–0·02 to 0·05–0·06 (the value for the Oxford strain was 0·01–0·02). Similarly, eleven strains of this group were directly tested for penicillinase production and all gave a negative result; the percentage inhibited distances of the heavy streaks lay within the range 59–68 %.

The ten *unlysed strains*, on the other hand, were all relatively resistant to penicillin; of nine tested by light streaks against the linear penicillin source, the range of percentage inhibited distances lay between 52 and 63 %. Eight strains (including the one not tested by streak) of this group whose cell sensitivities were accurately determined by the plate-dilution test, gave values of 0·2–0·4 to 0·6–0·8 unit/ml. Penicillinase production was tested directly in eight strains; all were positive. The percentage inhibited distances with the heavy inocula varied in nine strains from 7 to 53 %.

Additional observations on very resistant strains. Seven further strains were fully tested which differed from the above strains in the lysis test, since heavy streaks grew right up to the penicillin source, so that the junction of free and inhibited growth could not be observed. All these seven strains produced penicillinase, and it is thought that they produced sufficient enzyme to enable them to grow freely in the highest concentration of the drug which they encountered on the plate. Four strains were tested by the plate-dilution method; the cell sensitivities varied from 0·6–2·0 to 1·0–3·0 units/ml. In six instances the percentage inhibited distance was determined with light streaks, and lay in the range 40–63 % (five within the range 46–63 %).

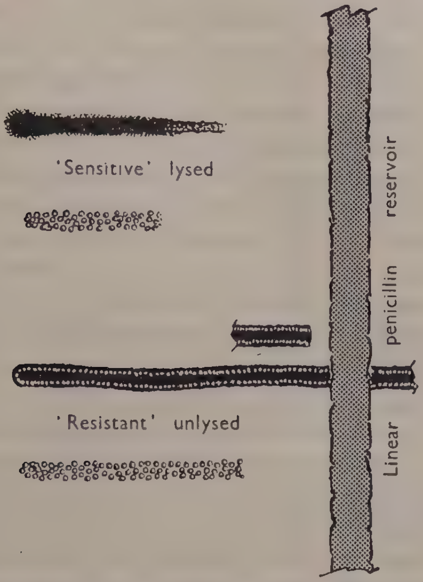

Fig. 3. (Scale $\frac{2}{3}$). Heavy and light streaks of sensitive and resistant strains of *Staph. aureus* evenly sown at right-angles to a linear penicillin reservoir. Lysis is shown by the sensitive strain (which does not produce penicillinase) in the 'comet-tail' lying between normal growth and the inhibited portion of the streak. It is best seen in the heavy streak. The resistant, penicillinase-producing strain may grow right up to a reservoir of low concentration, but when inhibited at a distance from a higher concentration (as in the short heavy, streak immediately above the 'resistant' unlysed streaks) shows no lysis.

DISCUSSION

In this small sample it seems that there is a division of staphylococci into two groups: (*a*) those whose cells are sensitive to penicillin, do not produce a penicillin-destroying enzyme, and are lysed by penicillin; (*b*) those whose cells are resistant to penicillin, are not lysed by it and produce a penicillinase. The second group of course includes only those staphylococci naturally tolerant of penicillin, and not those trained to tolerance by the artificial selection of variants, which are usually of low virulence and are uncommon in clinical lesions.

The failure to detect any differences in the biochemical and cultural characteristics of the two groups agrees with Barber's (1947*b*) report that such tests fail to distinguish penicillinase-producing strains from those which do not.

There is a large number of tests now in use for the routine clinical determination of penicillin sensitivity (tolerance). Many of these depend on observing the growth of cultures in relation to a penicillin source on an agar plate, and applying an arbitrary distinction between those considered sensitive and those classed as resistant. It is, of course, realized that these tests do not usually allow for the possible effect of penicillinase upon the result of the test, and so in using such techniques one is in doubt of what is really being assessed.

Spink *et al.* (1944), Luria (1946) and Barber (1947*b*) showed that the experimental value obtained for the penicillin tolerance of a penicillinase-producing staphylococcus depended on the size of the inoculum, whereas with a non-producer of penicillinase the inoculum size did not affect the tolerance value. In any measurement depending on the diffusion of penicillin through agar, the depth of the agar (Hayes, 1945), the composition of the batch of medium (Pulvertaft, Greening & Haynes, 1947), the volume of solution in the penicillin reservoir (Grenfell, Legge & White, 1947) and perhaps other factors will affect the value obtained, irrespective of the concentration of penicillin against which the organism is tested. In the present work precautions were taken to standardize the inoculum (light streaks) for the penicillin tolerance tests, and to use so small an inoculum that the effect of penicillinase would be inappreciable.

The tenfold difference observed between the cell-sensitivity ranges of the two smaller groups of strains tested by dilution plates is of the same order as that found by Bondi & Dietz (1945), who examined penicillinase production and penicillin tolerance (also by penicillin dilution plates) in 115 strains of staphylococci; although fairly heavy inocula were used, it is probably significant that their strains also fell into two groups whose tolerance ranges did not overlap. All the more tolerant strains (16) and none of the less tolerant (99) produced the enzyme.

Evidence on the production of penicillinase was also thought to be provided by the percentage inhibited distances of the heavy streaks. This value was much less in the strains known to produce the enzyme than in those not producing it, and the greater diminution was thought to be due to it. This agrees with the findings of Spink *et al.*, of Luria and of Barber quoted above. The strains which did not produce enzyme also showed some diminution of the percentage inhibited distance in heavy streak; this is apparently at variance with the findings of those authors. It is therefore possible that these very dense streaks contained enough more tolerant cells to have produced a visible growth nearer to the penicillin source than was possible in the corresponding light streaks. Numerical data on this point will be presented elsewhere (Ingram, Foxell & Armitage, unpublished). The above finding is in accord with that of Gilson & Parker (1948), who reported an increase in the tolerance range of staphylococci not producing penicillinase from 0·008–0·12 unit/ml. to

0·016–1·0 unit/ml. when the inoculum was increased by only a hundred-fold. By comparison, the same inoculum increase with the penicillinase-producers gave an increase in the tolerance range from 0·06–4·0 units/ml. to 0·06–500 units/ml.

If the results of the present investigation are confirmed, either penicillin lysis or penicillinase production (as suggested by Barber, 1948) would be satisfactory as single criteria of the penicillin sensitivity of staphylococci for ordinary clinical purposes. These characteristics are not quantitatively determined by the arbitrary conditions of a given plate, as is, say, the 'inhibited distance' from the penicillin source. Barber (1948) has pointed out that the presence or absence of the ability to destroy penicillin is of much greater clinical significance than the exact sensitivity of the strain.

A high concentration of penicillin should be present in the diffusion source if observations are to be made on the junction of normal and inhibited growth in the case of powerful penicillinase producers.

Grateful acknowledgement is made to Prof. W. G. Barnard and the Council of St Thomas's Hospital Medical School for a Bursary during the tenure of which this investigation was carried out; to Dr J. Bamforth for laboratory facilities; to Dr V. D. Allison for phage typing and Dr A. W. H. Foxell for penicillinase tests; to P. Armitage, Esq., of the Medical Research Council Statistical Research Unit, for kindly performing the computations for the strip-to-strip reproducibility test of the strip-soaking investigation; to Dr N. G. Heatley, Dr Mary Barber and Dr R. L. Vollum for helpful criticism of MS.; to Dr W. A. R. Thomson of Boots Pure Drug Co. for the gift of penicillin; to James A. Jobling Co. for the gift of glass capillaries; to the late Mr Radford, and to Miss Whitley and Miss King for technical assistance, and to the Hospital carpenters and engineers, especially Mr Brignall, who constructed the base and cover of the apparatus used for the 27 × 27 cm. agar slab.

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Cell Structure in Relation to the Gram Reaction as Shown During Lysis of *Bacillus subtilis*

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SUMMARY: The cell wall of *Bacillus subtilis* was shown to lie externally to the Gram-positive staining area of the cell. Conversion of these bacteria to the Gram-negative state, whether natural or induced, did not necessarily result in a decrease in cell size. When a decrease occurred it was correlated with the disappearance of an 'ectoplasm' staining area and with the inability of the residual cell substance to be converted to the Gram-positive state with magnesium ribonucleate.

Cell autolysis proceeded as follows. First the cell lost magnesium ribonucleate and was therefore Gram-negative, the ectoplasm and cell wall staining areas were retained, and the cell could be reconverted to the Gram-positive state by exposure to magnesium ribonucleate. The size of the cell was normal. Next, the ectoplasm and cell wall staining areas disappeared. This was correlated with an inability to be reconverted to the Gram-positive state and with a smaller size. Finally, cell autolysis was completed and the cell disappeared.

Gram-negative species were shown to possess an ectoplasm staining area which differed from that of normal Gram-positive cells since it could not be made to stain Gram-positive by exposure to magnesium ribonucleate. The ectoplasm staining area of Gram-positive cells was shown not to be in itself the reason for Gram-positive staining. However, the presence of this area correlated with the cells' ability to be reconverted to the Gram-positive state by magnesium ribonucleate, and thus it is probably important in the Gram reaction.

It has long been established that lytic procedures result in the conversion of Gram-positive bacteria to a Gram-negative state. This conversion has been shown to result from the action of autolytic enzymes within the dead or dying cell (Dubos, 1937; Jones, Stacey & Webb, 1949), crystalline ribonuclease (Bartholomew & Umbreit, 1944), bile (Henry & Stacey, 1943), acids and alkalis (Deussen, 1920), and many other agents. The presence of Gram-negative forms in cultures of Gram-positive bacteria is thought to be due to the autolysis of the dead cells present (Dubos, 1937; Frazier & Boyer, 1934). In a culture of *Bacillus subtilis*, the cells which stain Gram-negatively are frequently observed to be smaller than those which stain Gram-positively. This observation has never been adequately explained. Some have interpreted the larger size of the Gram-positive organisms as being caused by the dye-iodine complex adhering to the outside of the cells. Others have interpreted this as an outer Gram-positive layer, and an inner Gram-negative layer, the outer layer having been lost by the Gram-negative forms (Gutstein, 1924, 1925; Eisenberg, 1909; Churchman, 1927, 1929).

Gutstein (1924, 1925) and Eisenberg (1909) claimed to have stained an ectoplasm layer in bacterial cells, which may be the same as the Gram-positive cortex of Churchman (1927). The work of Gutstein and of Eisenberg can be easily reproduced, and an outer protoplasm layer is stained by these methods.

A definite area exterior to the protoplasm can be stained by any one of the many cell-wall stains which have been published (Dyar, 1947; Knaysi, 1941; Welshimer & Robinow, 1949). As far as the present authors are aware, no attempt has been made to correlate the conversion of a Gram-positive cell to the Gram-negative state (sometimes producing cells which appear much smaller) with the state of the cell wall and ectoplasm staining areas.

The following paper reports the results of experiments designed to follow staining changes occurring in the cell wall and ectoplasm staining areas during the conversion of *B. subtilis* cells into a Gram-negative state.

MATERIALS AND METHODS

Our method consisted of staining cells by Gram stain and by special stains for cell wall, ectoplasm, etc. Wherever possible, the various staining methods were applied in succession to a single heat-fixed bacteriological slide and the results recorded by photo-micrography after each stain used. It was usually possible to follow one bacteriological field through several different staining methods.

Organism. The cells from a 12 hr. nutrient agar slant of *B. subtilis* grown at 25° were suspended in distilled water and several hundred thin smears were made. All slides were heat fixed.

Gram stain. Hucker & Conn's (1923) modification was used throughout. Measurements were also made using the Kopeloff & Beerman (1922) modification for purposes of comparison and no differences were found between the size of the cells stained by the two methods.

Cell wall stain. Dyar's (1947) cell wall stain was used. Three drops of 0·34 % cetyl pyridinium chloride were applied to the smear and 1 drop of saturated aqueous Congo red was mixed with this on the slide. The slide was then washed in water and dried.

'*Ectoplasm*' *stain* (see Discussion). The Gutstein (1924) modification of Eisenberg's (1909) method was used. Gutstein stated that this procedure stained the 'membrane and external part of the cytoplasm'. The smears were mordanted with 5 % aqueous tannic acid for 5 min., and then stained with 1 % aqueous malachite green for 1 min. There can be no doubt that this procedure stains an area, interior to the cell wall area, which appears as a deeply stained ring around the inner area of the protoplasm. The exact nature of this stained area has never been determined. In this paper we are interested in it simply as an area which can be differentiated by staining methods.

Ribonuclease digestion. The method of Bartholomew & Umbreit (1944) was used. Cells were suspended in distilled water and autoclaved. Crystalline ribonuclease solution (4 mg./100 ml. distilled water) was combined in equal volume with the autoclaved cell suspension and the mixture incubated in a water-bath at 45° for 1 hr.

Bile digestion. This digestion was conducted as outlined by Henry & Stacey (1943). Cells were placed in 2 % bile and held at 60° overnight or longer.

Replating. The method of replating used was that of Henry & Stacey (1943). The enzyme-digested cells (Gram-negative cytoskeletons) were washed by centrifugation in distilled water, resuspended in 1 % magnesium ribonucleate plus 0·1 % formaldehyde for 5 min., and then smeared on slides. Most of these cells were then Gram-negative.

Photographs. All photographs were taken with a Bausch & Lomb apochromatic objective 90 ×, N.A. 1·30, and a 20 × eyepiece. The pictures were enlarged to 3600 × for reproduction here.

Measurements. Heat-fixed cells were used for all measurements. Cell widths were measured with a calibrated filar micrometer and by direct measurements of enlarged photographs. The filar measurements represent the average of fifty to eighty cells and the photographic measurements are the average of all cells appearing in a single photograph (usually twelve to twenty-five cells).

General. Cells treated in various manners were stained, measured, and photographed; untreated slides were stained by Gram's method and suitable fields where Gram-negative cells occurred photographed. In cases where a series of photographs were made of the same field, the smear was decolorized between stains with ethanol or acetone, restained, and photographed again. It was found to be immaterial whether the Gram stain was applied before the cell wall stain, or vice versa, with or without an intervening stain with a single dye, as the morphology and size were not changed. However, after the ectoplasm stain was performed, no further restaining could be done as the mordanting process caused a permanent change in the cells.

RESULTS

Our first experiments were conducted to convince ourselves of the validity of Dyar's cell wall stain. In plasmolysed cells of *B. cereus* the cell wall remained intact with the cytoplasm visibly shrunk away from it. There can be no doubt that Dyar's procedure stains a structure exterior to the protoplasm, and that this structure is at least in part the cell wall. The relationship of the capsule to this staining procedure has never been convincingly shown, but this point is not important as far as the following experiments are concerned.

Dyar's cell wall stain was compared to the Knaysi (1941) stain and similar results were obtained. Welshimer & Robinow (1949) had previously compared their stain with that of Dyar, and found it to be similar. Therefore the Dyar stain is comparable to other well-known cell wall stains.

Next, an extensive series of cell measurements was made. Comparisons were made between widths of cells stained by the various procedures used, and of cells in various stages of conversion from Gram-positive to Gram-negative. The results are presented in Table 1.

Figs. 1–4 of Pl. 1 can also be used in interpreting these data. In these figures cell 1 is a small Gram-negative cell, cell 2 is a partially converted Gram-positive cell, and cells 3–5 are normal Gram-positive cells. Fig. 1 represents a Gram-stained smear. Figs. 2–4 represent the same field stained respectively with crystal violet, Dyar's cell wall method, and with Gutstein's ectoplasm stain. Much of the following can be verified by applying calipers to the photographs.

The Gram-positive cells average 0·87 μ. in width, while the width between the inner areas of the cell wall stained material was 0·71 μ. A visual comparison of

Table 1. *Average cell measurements as determined by filar micrometer (fifty to eighty cells) and photomicrographically (at least twenty cells)*

	Measurement (μ.)	
	Photomicrographic	Filar
Crystal violet alone, total width	0·90	0·98
Safranin alone, total width	0·74	0·72
Acid fuchsin, total width	0·74	—
Crystal violet and iodine, total width	0·92	1·04
Gram-stained, Gram-positive form	0·87	0·83
Gram-stained, small Gram-negative form	0·37	—
Gram-stained, large Gram-negative form	0·87	—
Gram-stained, ribonuclease-produced Gram-negative forms	0·75	0·76
Enzyme-produced Gram-negative forms, restored to Gram-positive state with magnesium ribonucleate (digested with cryst. ribonuclease)	0·85	0·86
Cell wall stain (Dyar's). Total outside width	1·29	1·14
Cell wall stain (Dyar's). Width between inner cell wall limits	0·71	—
Ectoplasm stain. Total outside width	0·81	0·80
Ectoplasm stain. Width between inner ectoplasm limits	0·44	—

figs. 1 and 3 shows that the Gram-positive cells fit within the cell-wall area. It is obvious that the larger size of the Gram-positive cells is not due to a dye-iodine precipitate on the outside of the cell and the Gram-positive material must lie within the cell-wall area. It is also obvious that Gutstein's ectoplasm staining procedure stains an area mostly within the cell wall, and corresponding to approximately the outer half of the protoplasm material. In this, and many other strains studied, the Gram-negative cells seemed to be of two types. Some were small and averaged 0·37μ., most were large and averaged 0·87μ. Some intermediates were observed. Cells converted to the Gram-negative state by the action of crystalline ribonuclease were nearly the same width as normal cells, unless the digestion was continued over a prolonged period of time. The normal-sized Gram-negative cells were still of normal width when replated with magnesium ribonucleate. The obviously small Gram-negative forms could not be replated with magnesium ribonucleate. The small difference in size between the crystal violet stained cells and those stained with safranin and acid fuchsin is probably without significance.

An extensive study was made of the correlation of Gram-positive to Gram-negative conversion with the appearance of the areas stained by the cell wall and ectoplasm staining methods. Although a large number of pictures were taken, the essential results can be represented by figs. 1–12 of Pls. 1 and 2. The Gram-negative cells shown in these pictures were all the result of natural autolysis in a culture. Cells 1–5 in figs. 1–4 show the following points: cell 1 is a small Gram-negative cell in a chain of Gram-positive cells. It no longer is differentially stained by either the cell wall or ectoplasm methods, and it is distinctly smaller in size than the other cells shown. Although the cell wall is not clearly stained, as in the other cells, this staining procedure does result in a wider cell than when the Gram stain was used. Cell 2 is weakly Gram-positive and has lost some of its ectoplasm staining material and cell wall staining material. The rest of the cells in the field are normal.

Many observers of a cell wall and ectoplasm stain preparation report that to them the stains appear to be staining similar structures. A glance at the adjacent ends of cells 4 and 5 as seen in figs. 3 and 4, shows clearly that different structures are stained by these two methods.

The Gram-negative cell marked with the arrow in figs. 5–8 (Pl. 1) shows that a cell may stain Gram-negatively and yet retain the ectoplasm and cell wall staining material. This cell appears the same in width as the normal Gram-positive cells. The cell marked in figs. 9–12 (Pl. 2) shows a Gram-negative cell that still possesses cell wall staining material, but it does not possess ectoplasm staining material. The loss of this ectoplasm staining material has resulted in a cell which is visually smaller than the other cells, when stained by safranin, crystal violet, or the ectoplasm stain. Reduction in apparent size would then seem to correlate with the presence or absence of ectoplasm staining material.

Although the above experiments were conducted on naturally occurring autolysed cells, similar results were obtained in experiments using crystalline ribonuclease and bile. On conversion to the Gram-negative state with ribonuclease the ectoplasm and cell wall staining areas were still present, and could be restored to the Gram-positive state with magnesium ribonucleate. The cell wall and ectoplasm staining areas remained demonstrable unless the enzyme digestion was greatly prolonged, in which case factors other than the action of ribonuclease were responsible for the final disintegration of the cell. Bile was also used, with the same results except that it destroyed the ectoplasm and cell wall staining areas much more rapidly than the other methods studied.

Figs. 13 and 14 (Pl. 2) are presented to show that Gram-negative cells also have ectoplasm staining areas. This is in agreement with the earlier reports of Eisenberg (1909).

DISCUSSION

In this paper the word 'ectoplasm' has been used to indicate the area stained by Gutstein's 'ectoplasm' stain. Unfortunately the nature of this area is not known, and this has resulted in much confusion as to the meaning of this word when applied to bacterial cells. It is an area which is chemically differentiated by the staining method used, but the reason for this differentiation is unknown.

Churchman (1927, 1929) has explained the small size of the Gram-negative cells in a culture of Gram-positive bacteria on a morphological basis. He claimed that all Gram-positive bacteria consisted of a Gram-negative medulla and a Gram-positive cortex which was lost on conversion to the Gram-negative state and which resulted in a smaller cell since only the Gram-negative medulla remained. The present work does not necessarily confirm the concepts of Churchman. In the first place, the areas differentiated might simply be chemical layers and not actual morphological structures as claimed by Churchman. Secondly, we have shown that cells may be Gram-negative and still be of their original size, and this would be impossible by Churchman's concepts. Thirdly, we have not shown that the material left in a small Gram-negative cell

(Churchman's medulla) is actually Gram-negative in an intact and normal cell. The autolytic procedures by which the small Gram-negative stages were obtained, would also have had a considerable effect on the residual material.

It is apparent that if magnesium ribonucleate cannot be replaced on to the small Gram-negative forms, then the ectoplasm staining substance might well be necessary for magnesium ribonucleate to produce the Gram-positive state. If so, then this would mean that the essential Gram-positive materials must be located in the outer layers of the protoplasm. However, the ectoplasm staining material is not in itself responsible for the Gram-positive complex. This is indicated by its presence in Gram-negative cells and by the fact that Gram-positive cells converted to the Gram-negative state may still possess ectoplasm staining material.

Our explanation of the results presented in this paper is as follows. The cell wall is not stained by any of the usual staining procedures including the Gram stain, and it has been shown that the Gram-positive staining material lies interior to the cell wall material. The protoplasm can be differentiated into two layers by the method of Gutstein. On conversion of a Gram-positive cell to Gram-negative by autolytic or enzyme digestion, the first step is the release of magnesium ribonucleate (Bartholomew & Umbreit, 1944; Dubos, 1937; Henry & Stacey, 1943; Jones, Stacey & Webb, 1949). The cell now stains Gram-negatively and has intact all areas stained by the cell wall, ectoplasm, and ordinary staining methods. Magnesium ribonucleate can be recombined with the cell at this stage, and the now Gram-positive cell still has a normal width. Further digestive action destroys the ectoplasm and cell wall staining areas, the cell can no longer be reconverted to the Gram-positive state, and it is considerably smaller than the normal cell. Failure of the residual material to take the magnesium ribonucleate and hence stain Gram-positively, indicates that the ectoplasm staining layer plays an important role in the Gram-positive property. Finally, the process of autolysis or digestion may be completed and the cell may disappear. It is not clear whether the failure of the cell wall to stain at this later stage was due to its absence, or simply to a change in its chemical characteristics.

The ectoplasm staining area of normal Gram-negative bacteria deserves additional comment. This material must be considerably different from that in Gram-positive cells because the former is incapable of uniting with magnesium ribonucleate to produce the Gram-positive state (Bartholomew, 1946). This difference may well be due to the types of protein present in this area, and this difference may be that observed by Henry, Stacey & Teece (1945).

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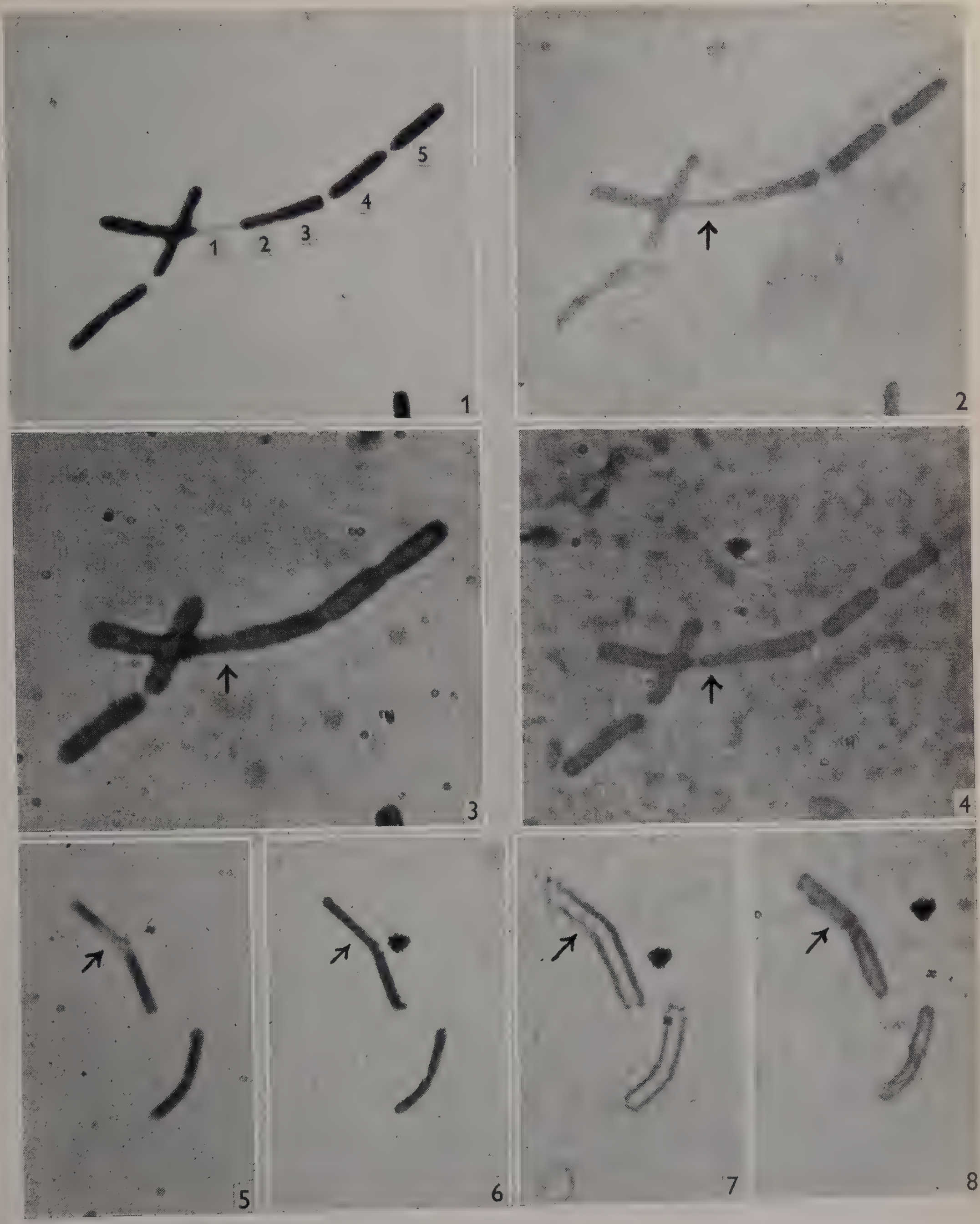
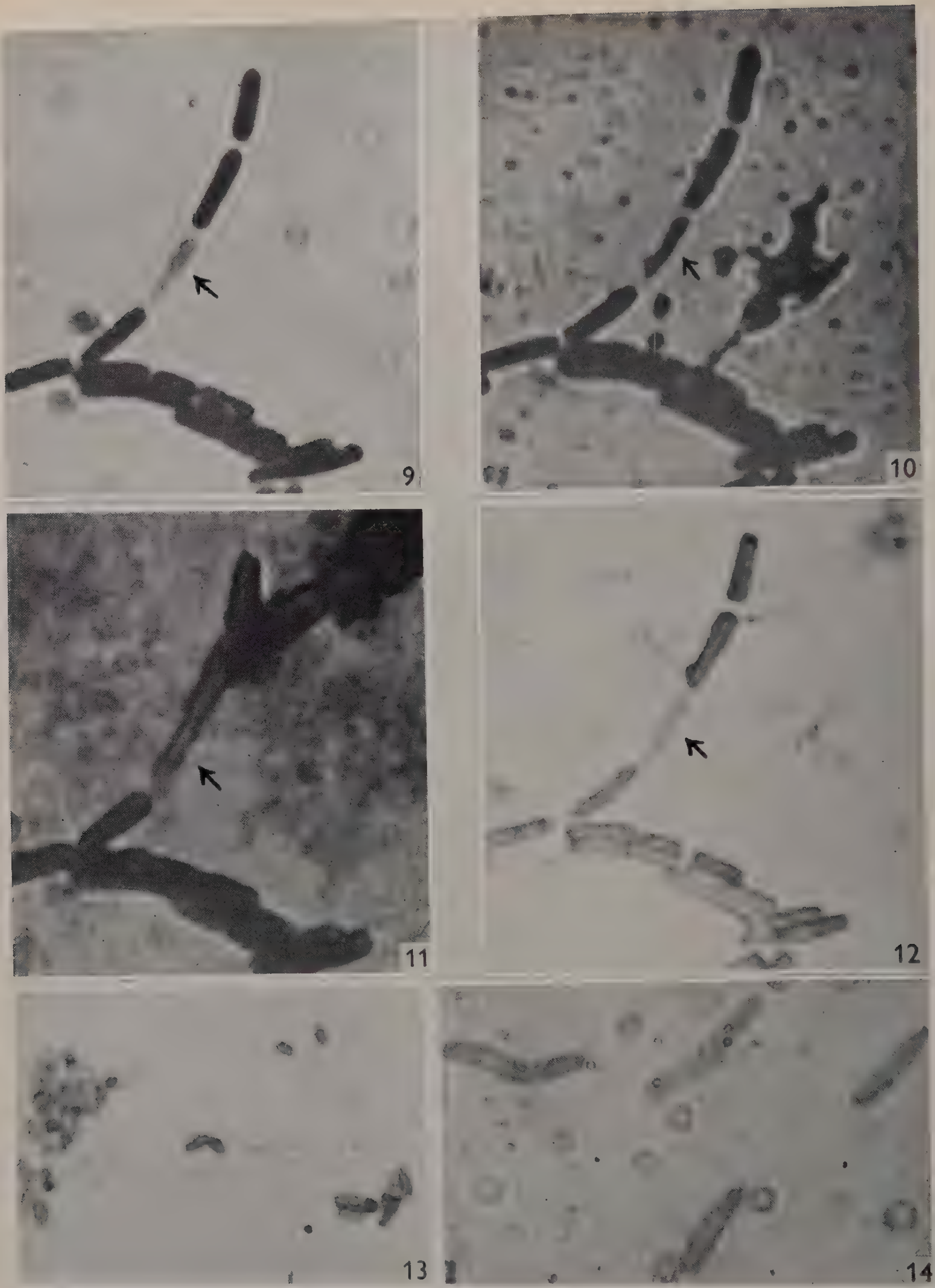


Figs. 1–8

J. W. Bartholomew and T. Mittwer—Cell structure in relation to Gram reaction. Plate 1



Figs. 9–14

J. W. Bartholomew and T. Mittwer—Cell structure in relation to Gram reaction. Plate 2

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EXPLANATION OF PLATES

All figures represent a magnification of × 3600.

PLATE 1

Figs. 1 and 5. *Bacillus subtilis*, Gram stain.
Figs. 2 and 6. Same fields, stained with crystal violet only.
Figs. 3 and 7. Same fields, stained with Dyar's cell wall stain.
Figs. 4 and 8. Same fields, stained with Gutstein's ectoplasm stain.

PLATE 2

Fig. 9. *Bacillus subtilis*, Gram stain.
Fig. 10. Same field, stained with crystal violet only.
Fig. 11. Same field, stained with Dyar's cell wall stain.
Fig. 12. Same field, stained with Gutstein's ectoplasm stain.
Fig. 13. *Pseudomonas putida*, stained with Gutstein's ectoplasm stain.
Fig. 14. *Spirillum* sp. stained with Gutstein's ectoplasm stain.

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A Genetic Approach to Variation in Influenza Viruses

1. The Characters of Three Substrains of Influenza Virus A (WS)

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SUMMARY: The three virus types derived from the classical WS strain of influenza virus can be distinguished by a relatively wide range of 'marker' characters, which are available for the recognition of virus characteristics in studies of variation and recombination. Despite their common origin the three WS strains have diverged very markedly one from another. Each has unique characteristics: NWS its absence of enzymic and indicator activity, its neurotropism; WSE its firm non-eluting adsorption to red cells; WSM its extremely heat-resistant haemagglutinin. From the point of view of the general pathology of virus infection the neurotropic character imposed on an influenza virus is the most important of the variant qualities.

A long experience of work with influenza viruses impresses one very strongly with the importance of variation both in epidemiological phenomena and in the laboratory manipulation of the virus. In this laboratory much attention has been directed to the initial variation by which influenza virus A, isolated directly from the human patient, is converted to forms that can multiply in the allantoic cavity of the chick embryo—the O–D change of Burnet & Bull (1943). This change appears to be due to mutation and selective overgrowth of the variant rather than to any direct influence of the environment on the multiplying virus population.

In recent years rapid progress has been made in developing a genetic approach to variation in bacteria (Avery, McLeod & McCarty, 1944; Tatum & Lederberg, 1947) and in bacterial viruses (Delbrück & Bailey, 1946; Hershey & Rotman, 1948). In both groups it was possible to demonstrate recombination of characters in the sense that when two strains, each with two or more 'marker' characteristics are mixed under conditions where multiplication of both can take place, a certain proportion of 'recombinants' can be subsequently isolated. If two strains designated by their marker characters AB and CD respectively, give rise to a proportion showing AD or BC characters and if under similar conditions AB or CD alone never give rise to such types, then it is legitimate to consider that recombination has occurred. Amongst the bacterial viruses studied by Delbrück & Bailey (1946) recombination is only demonstrable between strains of the same general type, e.g. amongst the morphologically similar even-numbered members of the T series of coliphages. Only under such conditions does it appear to be possible for two or more virus particles to infect a single host bacterium.

Interest in the possibility of a similar genetic approach to the influenza viruses was stimulated in the course of work with Stuart-Harris's neurotropic variant of the strain WS (influenza virus A). These experiments were concerned with the possible effect of infection on the haemagglutinin-inhibitor content of mouse brain. It was then realized that there were available in the laboratory three strains, all derivatives from the original WS of Andrewes, Laidlaw & Smith (1934) which had well-defined easily demonstrable differences and which, by analogy with bacterial viruses, should offer an opportunity to test whether recombination of characters could occur in influenza viruses.

In designing experiments of this type it became clear that a primary requirement was the use of pure clones of virus. Most of the marker characters that might be used were *in vitro* reactions shown by fluids containing high concentrations of virus particles. Typical examples are the capacity to agglutinate red cells of different species and the temperature at which this power to agglutinate is destroyed. A number of other markers are related to the enzymic function of the viruses in regard to soluble or cellular mucoids. All such tests reflect the activity of a large population of virus particles, and it is obviously desirable to ensure, as far as possible, the uniformity of the individuals making up that population.

By analogy with all other work on variation in micro-organisms we can feel confident that whenever a large population of virus particles develops from a small uniform inoculum, this population will contain a certain proportion of mutant forms. For *in vitro* tests of the type used in influenza virus work any proportion of mutants in the population below 1 % can probably be neglected. The presence of the mutants becomes important when successive transfer provides an opportunity for one or more of them to outgrow and replace the original form. In genetic work the necessity is to retain each significant type in a constant condition so that whenever required a clone of virus (in the form of an infected egg fluid) with consistent properties can be prepared.

This can readily be accomplished provided we are content to obtain only the predominant form, the type present in greatest numbers, in the material under study. All the evidence suggests that when a virus suspension is serially diluted until infection results in only a small proportion of highly susceptible hosts, those infections are initiated by a single virus unit. This holds particularly where an all or nothing response of the host occurs, as in chick embryo infections with well-adapted influenza virus strains. Pure clone technique with influenza viruses depends, then, on diluting the starting material till it is slightly below the 50 % infective level and on examining several of the fluids obtained from embryos infected by such a dilution. If these fluids are uniform in their relevant characteristics any one of them may be taken as representing a pure clone of virus, subject of course to the qualification that some production of mutants may have occurred by the time the virus is harvested.

Minor components of a virus population cannot be isolated at will. It may be possible to provide an alternative type of host cell which will favour the multiplication of a minority variant sufficiently so that it will overgrow the original predominant type and be isolated by the limit-dilution method. Exposure of a population to graded doses of destructive agents, heat, ultraviolet light, antiserum, etc., may also allow survival of variant forms initially in a minority. Both methods, however, allow the isolation only of mutants of types appropriate to the different techniques. Transfer of a mixture of clones to a new host may not necessarily allow the immediate demonstration of a mutant pathogenic for the new host even when such a mutant is present. The presence of an excess of the non-pathogenic form may 'interfere' with the multiplication of the pathogenic minority so that no lesions result and no isolation is possible.

The present work represents essentially an application of the principle of pure clone technique to a study of the influenza strains that have been derived from the classical WS strain and particularly the neurotropic variant developed by Stuart-Harris (1939). This strain is unique amongst existing influenza viruses in being able to multiply and produce fatal encephalitis in the mouse brain. This provides a specially valuable marker for recombination studies. This first paper deals with a preliminary study of the characteristics of pure clones of the three established variants of WS.

MATERIALS AND METHODS

It will be convenient to include here a general account of the methods used throughout the experiments reported in this paper and the three subsequent papers, leaving only special aspects of technique to be included in the latter.

The virus strains used were the standard strains maintained in this laboratory as infected allantoic fluids stored in a dry-ice refrigerator. Seed virus was prepared from embryos infected with such material and stored with the addition of glycerol to 33 % at −10°. Experimental virus as used in all tests was in the form of allantoic fluid harvested after 2 days at 35° from eggs inoculated at 11 days' incubation with 0·05 ml. of 10^{-4} or 10^{-6} dilution of seed virus. Throughout the whole series of experiments one or more of the three substrains of WS have been of central importance and a fairly full account of their origin is, therefore, desirable.

The strain WS was isolated at Hampstead in the spring of 1933 and given eighty-eight ferret passages before being transferred to mice early in 1934. Dried mouse-passage material was received from Dr C. H. Andrewes in 1937 and from this, strains WSM and WSE are derived.

WSM. A mouse-passage series was initiated with the mouse-lung material received from Hampstead in 1937. In 1940 the mouse virus underwent 100 passages by the amniotic route without showing more than trivial changes in character. Amniotic fluid from the 102nd passage was maintained as seed virus in a dry-ice refrigerator. It was revived by inoculating a dilution of 1/100 into embryos by the amniotic route and infected amniotic fluid from these, mixed with an equal volume of pure glycerol, was used as seed virus for the preparation of allantoic fluids.

WSE. The same dried mouse-lung WS received in 1937 was passaged on the chorioallantois as described by Burnet & Lush (1938); 56th passage virus was passed to the amniotic cavity and the strain kept in the dry-ice refrigerator with only one intervening passage between 1942 and 1949. This was revived and handled in the same fashion as WSM.

NWS. This was derived by Stuart-Harris (1939) from mouse passage WS, first by adaptation to the chorioallantois with the development of haemorrhagic brain lesions, followed by inoculation of embryo brain material to very young mice and serial passage. After twelve passages deaths occurred amongst mice and continued in subsequent passages. We received the strain from Prof. Stuart-Harris through Dr Alick Isaacs in the form of dried mouse-brain 'M103 dried 27/7/48'. When redissolved this was infective for chick embryos and mice. After one further allantoic passage the resulting fluid was titrated and showed two of four eggs infected at dilution 10^{-6}. One of these fluids was glycerolated and used as seed virus either directly or at one remove in all subsequent work.

Haemagglutinin titrations. Haemagglutinin tests were made by adding 1% fowl cells to equal (0·25 ml.) volumes of virus dilutions in saline and allowing them to sediment at room temperature. The reading is made from the pattern of deposited cells, the end-point being a standard degree of partial agglutination. All the other *in vitro* tests based on haemagglutination are derived in the simplest possible way from this basic technique.

Conversion of strains to indicator state. When a typical influenza B virus is heated to 55° for 30 min. it retains its power to agglutinate red cells apparently unaltered, but differs from the original active virus in two important respects. It no longer possesses the capacity of eluting from cells or showing other evidence of enzyme action, and its haemagglutinating action is readily inhibited by a variety of soluble mucoid materials. These two characters define what we have called the indicator state of these viruses. With many influenza A strains simple heating will not produce such a conversion, and as a routine Stone's (1949) method was used. Three volumes of allantoic fluid virus were mixed with one volume of 2% sodium citrate saline and one volume of borate buffer pH 8·5. This mixture was heated at 50° for 30 min. Occasional modification of this temperature for special reasons is indicated in the text.

In many instances it was desirable to determine whether simple heating converted a virus to the indicator state. This was done by preparing, on the results of preliminary titration, a dilution in saline containing five haemagglutinating doses (5AD) of virus. An ovomucin preparation made by Gottschalk & Lind's technique (1949) was diluted 1/5 in 0·85% saline, spun, and the supernatant used as starting-point for tenfold dilutions in saline. To 0·25 ml. of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of ovomucin an equal volume of indicator virus (5AD) was added, and after 30 min. at room temperature a further volume of 1% fowl cells. Full development of the indicator state would be shown by failure of agglutination with 10^{-3} and sometimes with 10^{-4} mucin dilution.

The position of a strain in the receptor gradient. It was shown by Burnet, McCrea & Stone (1946) that viruses could be placed in a linear order corresponding to the sequence with which red cells, treated with viruses or with the receptor-destroying enzyme (RDE) of the cholera vibrio, lost ability to be agglutinated by the viruses. The most convenient test for the position of a virus in this 'receptor gradient' is to test it with the standard viruses or red cells treated with graded amounts of RDE. Twofold dilutions of a crude stock filtrate containing 1200 units of enzyme/0·25 ml. were prepared in 4 ml.-volumes of calcium acetate saline pH 6·2 from 1/25 to 1/1600. To these 0·1 ml. of packed fowl cells was added and the tubes held at 37° for 30 min. Sodium citrate solution (0·4 ml. of 20%) was then added to each tube to stop all RDE action. The cells were washed and made up to 1 % suspension in 2 % citrate saline. Test and standard viruses diluted to 5 AD were then tested with each set of treated cells.

The characters of the three strains WSM, WSE and NWS

All these strains have a number of characters in common. They multiply freely in the allantoic and amniotic cavities of chick embryos, producing actively haemagglutinating virus. They give typical influenzal lesions on intranasal inoculation in mice. Serologically they are very closely related though, as will be shown later (Burnet & Lind, 1951) NWS seems to lack a minor antigenic component present in the others. They give rise to soluble complement-fixing antigen of A type. None of the strains shows visible filaments on dark-ground microscopy. They are presumably composed of spherical particles but they have not yet been compared by electron microscopy. They differ as follows.

Pathogenicity. The neurotropic strain NWS alone produces symptoms and fatal infection on intracerebral inoculation in mice. A typical allantoic-fluid virus gives fatal infections when diluted 10^{-5} but only a proportion at 10^{-6}. Deaths occur as a rule within 4–7 days and are usually preceded for 24 hr. by some signs of encephalitis. When the brain is removed from a mouse dying or just dead with typical signs, relatively large amounts of virus are present. As a routine the brain of each mouse tested was ground and suspended in 2 ml. saline. After centrifugation the supernatant fluid was infective to chick embryos to dilutions of 10^{-4} or 10^{-5}. Neither of the other strains produced any symptoms in mice after intracerebral inoculation with infected fluids either undiluted or diluted 10^{-2}. On the chorioallantois of 12-day embryos WSE and NWS produce similar well-defined foci at high dilution, but WSM produces lesions of weaker and more irregular character.

When inoculated intravenously in 12-day chick embryos the strains WSE and NWS produce fatal infections with characteristic massive haemorrhages in the brain and less regularly in other tissues, especially skin and lung. Even with very small inocula (dilution 10^{-5}) embryos after 3 days show brain and lung lesions, and both organs contain sufficient virus to allow 10 % extracts in saline to agglutinate fowl cells. When these extracts are heated to 65° and appropriately tested no inhibitor for haemagglutination by heated LEE virus is demonstrable. Extracts similarly prepared from uninfected organs have an inhibitor titre *c.* 60.

The strain WSM is not lethal within 3 days and only at relatively high concentrations (dilution 10^{-2}) is the lung infected sufficiently to show the presence of haemagglutinating virus and disappearance of virus inhibitor. No gross lesions are produced.

Haemagglutination. All these strains agglutinate human, guinea-pig, fowl and pigeon red cells readily and to comparable titres. Mouse cells are agglutinated less readily, WSE being the only one to produce complete agglutination; the other two give partial agglutination to a titre about one-fourth that shown with fowl cells. Rabbit cells are slightly agglutinated by WSE, not by the others.

In the cold (0°) fowl cells adsorb any of the strains but NWS somewhat less readily than the others. On prolonged treatment at 37° fowl cells are stabilized by WSM and less completely by NWS, with elution of virus. With WSE, however, virtually no liberation of virus occurs at 37° and the cells remain persistently unstable. As was first shown by Briody (1948) the enzymic action of WS strains is potentiated by Ca ions and inhibited by calcium deionizers such as citrate. Even in the presence of Ca ions, however, no elution of WSE is observed.

Table 1. *Position of WS virus strains in receptor gradient*

(The action is shown of five agglutinating doses (AD) of each virus on fowl red cells treated with excess of virus and in the case of WSE stabilized with immune serum, or treated with graded amounts of receptor destroying enzyme RDE corresponding to the dilutions shown.)

	Cells treated with excess of			Cells treated with receptor-destroying enzyme RDE					
				Dilutions of RDE					
Virus	NWS	WSM	WSE	1/50	1/100	1/200	1/400	1/800	1/1600
NWS	−	−	+	−	−	−	−	± ±	+ +
WSM	+	−	+	−	−	−	±	+ +	+ +
WSE	+ +	+	+ +	−	−	±	+ +	+ +	+ +

+ + = complete agglutination; ± ±, +, ± = degrees of partial agglutination;
− = no agglutination.

The order of the viruses in the receptor gradient is NWS, WSM and WSE. When fowl cells are treated with virus in the presence of excess Ca ions and in the case of WSE stabilized with immune serum, the reactions with different viruses are as shown in Table 1. In the same table are shown the reactions with cells treated as described under methods with graded amounts of RDE. The discrepancy with cells treated with WSE is due simply to the failure of receptor destruction by the virus. In relation to the other viruses of the receptor gradient all the WS strains come early in the series. There are the usual minor differences according to the agents used in treating the cells but with fowl cells the order observed is MU, NWS, NDV, WSM, MEL, WSE and LEE.

There are marked differences in the thermostability of the three haemagglutinins. In the experiment shown in Table 2 freshly harvested allantoic fluids were buffered by the addition of M/3 phosphate to give a final M/30 concentration with pH 7·35. These were heated for 30 min. at each of the temperatures shown. Haemagglutinin titrations were made and each fluid, diluted 1/10, inoculated into chick embryos. The striking thermostability of WSM haemagglutinin is to be particularly noted. The addition of phosphate buffer was probably unnecessary as in many other tests untreated fluids were heated for 30 min. at 54° and 62°. The lower temperature always decreased NWS haemagglutinin concentration to a very low level while 62° had practically no action on any WSM fluid. The heat resistance of infectivity for chick embryos is in the same order but as would be expected the differences cover a much smaller temperature range.

Enzymic activity and indicator state. The WS strains are all relatively weak in their enzymic action on ovomucin. When tested under the usual conditions

Table 2. *Thermostability of haemagglutinin and infectivity of WS strains heated for* 30 *min. at various temperatures*

Temp. (° C.)	NWS		WSE		WSM	
	HA	Inf.	HA	Inf.	HA	Inf.
Unheated control	400	·	1000	·	600	·
52	270	+ +	500	+ +	1000	+ +
54	4	− − −	350	+ +	800	+ +
56	< 2	·	170	− − −	650	+ + +
58	·	·	< 10	− − −	480	− − −
60	·	·	·	·	640	·
62	·	·	·	·	400	·
64	·	·	·	·	360	·
65·5	·	·	·	·	80	·

HA = Haemagglutinin titre; Inf. = Infectivity for chick embryos: each + represents an egg giving haemagglutination; − = no haemagglutination.

none of the three show any power to diminish its inhibitory titre against any of the indicator viruses. When, however, the ovomucin is diluted 1/50 instead of the usual 1/5 in calcium acetate saline, WSM and WSE both destroy the inhibitory action against H-WSE but not against the other indicators H-LEE and T-MEL (see Stone, 1949). NWS under these conditions has no demonstrable action.

A very sensitive test for enzymic activity has been described by Edney & Isaacs (1951). This depends on the well-known fact that normal allantoic fluid contains virus inhibitor, the behaviour of which is essentially the same as that of dilute ovomucin. Infective fluids of strain WSE harvested at 48 hr. and heated at 65° to destroy haemagglutinin are found to lack all inhibitors. This is also the finding with all other strains tested by Edney & Isaacs, but the influence of WSE is particularly striking because *in vitro* it does *not* destroy inhibitory action against strains H-LEE or T-MEL. The only exception to the rule that H-LEE inhibitors are always absent from an infective fluid of reasonable haemagglutinin titre is NWS. Fluids infected with NWS show practically normal content of H-LEE and T-MEL inhibitors but the H-WSE inhibitor is reduced or eliminated. One of the most delicate tests as to whether only NWS is present in an infected allantoic fluid is the estimation of H-LEE inhibitor after the fluid has been heated to destroy haemagglutinin. If it shows the normal value of 50–100 no other variant than NWS is present.

Alone amongst influenza viruses it has not been found possible to convert NWS into the indicator state. With all other WS variants heating in citrate at pH 8·5 for 30 min. at 50° converts them to a form, haemagglutination by which is inhibited by high dilutions of ovomucin and similar substances. A second test for identifying NWS is therefore to heat the fluid under these conditions and prepare a dilution to contain 5 AD of the modified virus. This is tested against tenfold dilutions of stock ovomucin solution. With the three viruses the usual findings are as shown in Table 3.

Table 3. *Conversion of WS viruses to the indicator state*

(Each virus was treated as described in the text by heating to 50° at pH 8·5 in the presence of citrate and 5 AD then mixed with an equal volume of the ovomucin dilutions shown. After 30 min. a third volume of red cells was added giving the degrees of agglutination shown.)

Treated virus	Ovomucin dilutions			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
WSM	–	–	–	+
WSE	–	–	–	++
NWS	±	+	++	++

++ = Complete agglutination; +, ± incomplete agglutination;
– = no agglutination.

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A Genetic Approach to Variation in Influenza Viruses

2. Variation in the Strain NWS on Allantoic Passage

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SUMMARY: When the strain NWS of influenza virus, which is adapted to multiplication in mouse brain, is submitted to allantoic passage in the chick embryo, it maintains its original character if passed at limiting infective dilutions. When passage is made with a low dilution of the same virus preparation the strain changes toward a more 'normal' character. These phenomena are regarded as providing formal proof that the changes are mutational in origin.

In the course of work on the O–D transformation of influenza virus A (Burnet & Bull, 1943; Burnet & Stone, 1945; Burnet, Stone, Isaacs & Edney, 1949) we have repeatedly found that the O form can be maintained on serial passage only if limit or near-limit infective dilutions are used. Unfortunately no record exists of confirmation of this finding from any other laboratory. We have consistently held that the phenomenon provides a formal proof that variation from O to D phase represents a mutation equivalent to a gene-mutation in higher forms, with selective overgrowth of the mutant, and is not a direct effect of the new environment on the virus population as a whole. The O–D change in influenza virus is admittedly difficult to study, entailing the inoculation by the amniotic route of large numbers of eggs and the detailed analysis of the haemagglutinating activities of each fluid and lung emulsion. It seems therefore desirable to present essentially similar evidence that a well-defined variant type of virus established in another host behaves similarly on passage in the allantoic cavity.

The argument is simply that if a strain in phase A on repeated passage by ordinary methods eventually gives rise to a strain showing different behaviour, phase B, one of two mechanisms may have been concerned in the change. The first alternative is that by repeated reproduction in a new intracellular environment the quality of the virus particle is progressively modified till it takes on the character of phase B. By modification we mean a process involving all or the great majority of the individuals in the continuously changing populations concerned, the change being a direct result of the new environment. A process of this type is postulated by Hinshelwood (1946) for the rapid development by certain bacterial strains of the capacity to ferment a sugar which they cannot initially attack. The second possibility is that amongst the mutants constantly being produced by the original strain there are some better fitted than the original to multiply in the new environment. It is these mutants (or further mutants derived from the primary ones) which eventually give to allantoic fluid virus the new B phase characteristics. A distinction between the two alternatives is possible when, as is usually the case, mutation is a relatively infrequent occurrence. In a number of instances the frequency with which a recognizable mutation occurs is of the order of once in every 10^5 or 10^6 life cycles of the organism concerned. If the conditions for viruses are broadly similar it follows that if one infective virus particle is introduced into the allantoic cavity a mutant is highly unlikely to arise until the original form has multiplied so extensively that there is no possibility of the mutant overgrowing it within the usual 44 hr. before the fluid is harvested. The great majority of virus particles in this fluid will therefore be of the original type. When dilution of 10^{-8} is used for the next passage no more than one to five infective particles will be transferred, all being of the original type; and if this process is continued, the same situation holds at every passage. On the other hand, when passage is made at low dilution so that the inoculum contains in addition to the original form a proportion of mutants capable of multiplying more rapidly, the fluids obtained will eventually be dominated by those mutants which grow most effectively under the experimental conditions.

If it is found that with repeated passage at limiting dilution (10^{-7} or 10^{-8}) a virus retains its original character, but with the same number of passages at a dilution of 10^{-2} gives rise to a recognizably different type, the change must be ascribed to mutation. The virus population obtained after the completion of the limiting dilution series is composed of virus particles which on the average are separated from the original virus particles used to initiate both series by a greater number of generations than those of the low dilution series. The limiting dilution virus has thus had *more* opportunity to be influenced by the intracellular environment than has the other series. A result of this type therefore rules out the possibility of the change being due to direct environmental modification.

The object of this paper is to show that such a situation can be demonstrated by passage of the neuro-WS strain of influenza A (Stuart-Harris, 1939) in the allantoic cavity of the chick embryo.

MATERIALS AND METHODS

The strain NWS (Stuart-Harris, 1939) has been described in the previous paper (Burnet, 1951) as well as the methods by which it can be characterized *in vitro* and *in vivo*. The passage series were commenced with virus two egg-passages removed from the dried mouse-brain material received from Prof. Stuart-Harris through Dr Alick Isaacs. Eggs at the 11th day of incubation were inoculated with 0·05 ml. of virus serially diluted in cold nutrient broth and incubated at 35°. As a matter of convenience two passages each week were made with 44 hr. incubation and one with 68 hr. The proportion of eggs showing haemagglutination by allantoic fluid was determined and selected positive fluids were used for detailed tests and for sub-inoculation. The high dilution passages were made at 10^{-7} and 10^{-8} dilutions, and a fluid from the highest positive dilution used for each passage. In the later passages only the 10^{-8} dilution was used. The 13th failed to cause infection in any of the eight eggs inoculated at 10^{-8} and this had to be repeated at 10^{-7}. This is the reason that there is one less passage in the high dilution series than in the low dilution series. In the latter, four eggs were routinely inoculated with a 10^{-2} dilution of the previous passage fluid. At various stages tests were made for heat stability of haemagglutinin, capacity to develop indicator state and to produce encephalitis in mice according to the techniques described in the previous paper.

RESULTS

Representative fluids from the 3rd, 6th, 10th and final (13th or 14th) passages were tested with the results shown in Tables 1 and 2. After fourteen passages at 10^{-2} dilution the last fluid of this series was tested at high dilution and the two positive fluids 53,594 and 53,596 obtained with a dilution of 10^{-8} from eight inoculated embryos were tested in detail against four fluids of the 14th passage of the high-dilution series. The fluids from the low-dilution series thus represent clones derived from two particles of types present in high concentration in the final fluid.

The points to be noted are that the high-dilution series have retained all the characteristics of NWS. In the low-dilution series there are two types of fluid.

Table 1. *Characters of high and low dilution passage series of NWS virus*

Passage		Allantoic fluid							
					Haemagglutinin titres				
No.	At dilution	Indicator titre	Inhibitor titre* of heated fluid		Original		Heated at 54° for 30 min.		Intracerebral pathogenicity for mice†
3rd	10^{-8}	60	48	80	600	700	<2	<2	·
	10^{-2}	60	48	48	600	600	<2	320	·
6th	10^{-8}	100	80	80	600	280	10	8	4, 5, 6, 8
	10^{-2}	1,500	28	24	350	500	50	12	5, 5, 6, 6
10th	10^{-8}	100	80	40	300	220	<2	<2	·
	10^{-2}	10,000	12	8	250	400	<2	<2	·

* Inhibitor titre against H-LEE of fluid heated at 62° for 30 min.

† Pathogenicity indicated by days to death of four mice inoculated intracerebrally with fluid diluted 10^{-2}.

Table 2. *Characters of re-isolated high and low dilution passage strains of NWS virus*

(The original haemagglutinin titres were in the range 200–800.)

Fluid	Haemagglutinin heated at 54° for 30 min.	Indicator titre	Inhibitor titre of heated fluid*	Intracerebral pathogenicity for mice†		
				10^{-4}	10^{-5}	10^{-6}
53,579 H	<2	80	24	5, 6, 6, 9	5, 6, 6, 6	5, 6, 7, 9
53,580 H	<2	40	28			
53,581 H	<2	80	64			
53,583 H	<2	40	32			
53,594 L	<2	20,000	2	6, 6, 6, 7	6, 6, 7, 7	6, 6, 7, 7
53,596 L	120	7,000	<2			

H = high-dilution passage, L = low-dilution passage.

* Inhibitor titre against H-LEE of fluid heated at 62° for 30 min.

† Intracerebral pathogenicity indicated by days to death of four mice inoculated intracerebrally with fluid diluted 10^{-2}.

Both differ from the original in being convertible into the indicator state and in having demonstrable enzymic activity. One fluid is resistant to heating at 54°, the other not. Both high- and low-dilution passage series have retained full virulence for mice by the intracerebral route. Evidence for the existence of other variants was sought by heating fluids to the temperature just capable of completely inactivating the normal form (54° for 30 min.). After three passages two fluids of each series were heated and then tested for infectivity undiluted and at a dilution of 10^{-2}. Positive fluids were studied in the usual manner.

Table 3. *Characters of fluids obtained from embryos inoculated with heated virus*

(Virus heated at 54° for 30 min.)

		Infectivity of heated virus		Allantoic fluid		
Series	Egg no.	Undiluted	10^{-2}	Haemagglutinin heated at 54° for 30 min.	Indicator titre	Inhibitor titre of heated fluid*
H	52,031	+ + +	+ − −	<2 <2	100, 80	64, 40
	52,032	− − −	− − −	.	.	.
L	52,039	− − −	− − −	.	.	.
	52,042	+ + +	+ + +	100	200	20

H = high-dilution passage; L = low-dilution passage.

* Inhibitor titre against H-LEE of fluid heated at 62° for 30 min.

It will be seen that fluid 52,042 contained much virus resistant to heating at 54°. The fluids tested were not from a limiting dilution so a further passage at high dilution was made. Positive fluids gave essentially the same characters as the first passage fluid shown in the table, but there was less residual inhibitor in the heated allantoic fluid. Both first- and second-passage fluids were inoculated intracerebrally in mice and produced no symptoms. It appears therefore that in embryo 52,042 a relatively heat-resistant and non-encephalitogenic variant had been able to multiply to a relatively high titre. On the other hand, the small proportion of virus that survived heating in fluid 52,031 of the high-dilution series gave fluids with the standard character of the original type.

DISCUSSION

These results show just as definitely as the experiments on the O–D phase variation, that influenza viruses are highly labile, changing in character with passage in a new environment, but equally that such change does not occur if transfers are consistently made at the highest infective dilution. As in the earlier case this provides a formal proof that the change in character on passage at low dilutions results not from 'adaptation' but as a result of the selective survival of mutant forms which eventually outgrow the original type.

We would suggest that just as any critical work on bacterial metabolism is always preceded by a replating of the strain concerned and choice of a typical colony, any study of virus properties should be carried out by using only clones of virus derived from an immediately preceding passage at the limiting infective dilution. The rationale is simply that mutation is a relatively rare occurrence and the product of multiplication of a single infecting particle in one host is, except under most unusual circumstances, bound to contain only a small minority of mutants. These are eliminated when passage is made at high dilution. When passage is carried on at low dilution there is a gradual accumulation of mutants in the stock the proportion of which will be determined essentially by their relative speed of multiplication in the environment. It seems obvious that an inoculum containing only one virus genotype will produce more uniform populations in the infective fluids harvested than an inoculum containing a variety of mutants. Chick embryos are by no means identical, one may well favour mutant x while another favours mutant y. A mixed inoculum may therefore give very different results in two different embryos (cf. eggs 52039 and 52042 above), but if a high dilution of a pure clone is used the only likely difference will be in regard to the actual concentration of virus attained by the time of harvesting.

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A Genetic Approach to Variation in Influenza Viruses

3. Recombination of Characters in Influenza Virus Strains Used in Mixed Infections

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SUMMARY: When the non-encephalitogenic strain of influenza virus A, WSM, was mixed with the influenza strain NWS in suitable proportion no encephalitis followed intracerebral injection of the mixture in mice. When the amount of WSM relative to NWS was decreased partial interference resulted and from four mice so inoculated, virus strains referred to as WS-NM were isolated, which appear to be recombinants. The NM strains were encephalitogenic for mice, though in varying degree, their haemagglutinin was almost as resistant to heat inactivation as that of WSM and they had at least one character (position in the receptor gradient) in which they differed from both the original strains.

In the first two papers of this series (Burnet, 1951 *a*, *b*) the characteristics of the strain NWS were described and the suitability of the WS variants for genetic studies was indicated. At various times we have made unsuccessful attempts to induce recombination of characters between influenza virus strains. Even on the assumption that recombination of characters of influenza viruses occurs freely whenever double infection of a single cell occurs, there are obvious technical difficulties in demonstrating its occurrence. In studies of this sort on bacterial viruses it is relatively simple to arrange that the relevant characters of *all* the progeny resulting from double infection of a single bacterium can be examined. With animal viruses one cannot work with single host cells, and the most that can be hoped is to produce a double infection and to obtain in the population of virus units resulting a proportion of recombinants amongst a majority of one or both the original forms. The existence of recombinants can then only be demonstrated if conditions can be so arranged as to favour their selective proliferation. Our object has been to concentrate on recombinants possessing the capacity to multiply in the mouse brain, so making possible the use of a highly selective environment. The difficulty has been of course to prevent the overgrowth of the original NWS necessarily present as one of the 'parent' viruses. This difficulty was overcome in a quite unforeseen fashion by using serial dilutions of the second 'parent' with a constant dose of NWS. With a suitable non-neurotropic strain, e.g. WSM, the higher concentrations will prevent multiplication of the neuro-virus by 'interference' and at the level where interference is just being overcome it is possible to isolate sharply distinct variants, which are most readily interpreted as recombinants.

MATERIALS AND METHODS

The virus strains NWS and WSM were described by Burnet (1951 *a*). They were used in the form of allantoic fluids from chick embryos inoculated at 11 days with 0·05 ml. of 10^4 dilution of a glycerolated seed virus stored at $-10°$.

Injections of 0·03 ml. into the right cerebral hemisphere were made in 4–5-week-old mice under light anaesthesia. Deaths were regarded as specific when the mouse had shown nervous signs, excitability or partial paralysis, on the day of or days before death and experiments were terminated after 12 or 14 days, deaths being very unusual after the 9th day. When indicated, brains of mice dead spontaneously or killed when moribund were removed aseptically, ground with alundum and each brain emulsified in 2 ml. saline containing 200 units, penicillin/ml. After being lightly spun the supernatant fluids were tested for their virus content by inoculation into the allantoic cavity of 11-day chick embryos. The eggs were tested for haemagglutinin after 44 hr. incubation at 35° and active fluids were studied in detail.

The principles of pure-clone study described by Burnet (1951*b*) were applied throughout, the practice being to work with limiting infective dilutions (L.I.D.) and to accept a fluid as representing a pure line if (*a*) it was derived from a L.I.D. and showed the same characters as other L.I.D. fluids from the same seed, and (*b*) on being tested by titration to the L.I.D., the L.I.D. fluids resembled each other and the parent fluid in all relevant characters. Serological work was confined to anti-haemagglutinin titrations with ferret sera from animals immunized by intranasal inoculation of active WSM or NWS virus. The animals were exsanguinated 14 days after inoculation.

Interference phenomena in mice inoculated with mixed NWS and WSM virus

When a fresh allantoic fluid of NWS with a haemagglutinin titre of *c.* 320 is inoculated intracerebrally in mice it produces fatal infections in a dilution 2×10^{-5}. Fluids of WSM usually of rather higher haemagglutinin titre produce no symptoms either undiluted or diluted 10^{-2}. When a mixture of the two viruses is used the result depends on their relative proportions. When WSM is in considerable excess signs fail to appear, and if such mice are killed at the time signs are appearing in mice receiving only NWS (i.e. 5–7 days after inoculation) no virus is isolated from the brains.

In Table 1 are shown the results of two experiments in which all the 'recombinant' strains subsequently discussed were isolated. In the first, two sets of mice were inoculated with serial dilutions of NWS; in one set the dilutions were mixed with an equal volume of diluent (10 % horse serum saline) in the other with an equal volume of dilution 10^{-1} of WSM. In the second experiment a standard amount of NWS (diluted 10^{-3}) was used with serial dilutions of WSM. These results, shown in Table 1, are characteristic of five other experiments with the same two viruses. When NWS diluted 10^{-3} was mixed with other viruses interference to an approximately equal extent was shown by influenza virus strains WSM, WSE, PR8, MEL, BEL, less-marked interference was shown by SW and none with mumps virus strain END or Newcastle disease virus.

Isolation of viruses from mice showing partial interference. When brains removed from mice dying from uncomplicated NWS infection are prepared as described and the supernatant fluids titrated, the end-point of infectivity is about 10^{-4} (0·05 ml. inoculum). With WSM alone virus can be isolated from mice killed on the 2nd or 3rd day but it is absent on the 6th day. Mice in which

Table 1. *Interference by WSM with NWS infection*

(The figures are the day of death of each of four mice, and the figures in brackets the day following that on which a sick mouse was killed for culture, i.e. the day on which death would probably have occurred spontaneously.)

	NWS dilutions				
(*a*)	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
+ Saline	4, 5, 6, 6	5, 6, 6, 7	5, 6, 7, 7	X, 6, 6, 6	S, S, S, S
+ WSM (10^{-1})	5, 5, 5, 7	5, 9, 9, (8)	X, (7), S, S	S, S, S, S	S, S, S, S

	WSM dilutions					
(*b*)	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Nil*
+ NWS (10^{-3})	S, S, S, S	S, S, S, S	5, 6, (8), (8)	5, 6, 6, 10	6, 6, 6, 10	6, 6, 6, 6

X = early non-specific death; S = survived for 12 days.
* Control no WSM dilution added.

mixed infection has produced no signs about this time have shown no virus. Even when paralysis or death results after infection with mixtures giving partial interference it is common to isolate no virus from the brain emulsion. This is particularly the case when death or gross symptoms are delayed beyond the 7th day. It is more usual to isolate virus only from the undiluted brain emulsion. From the two experiments shown in Table 1 a special study was made of the groups on the borderline of interference, NWS 10^{-3} + WSM 10^{-1} in A, NWS 10^{-3} + WSM 10^{-2} in B. Three of the mice in A and all four in B were investigated in detail with the results shown in Table 2. In this table, N signifies virus of the same character as NWS. This was the only type isolated (i.e. presumably present in largest amount) from mice inoculated with NWS only. N′ differed from N principally in being convertible to the indicator state, having more enzymic action and being slightly more heat resistant. NM strains were characterized primarily by the heat resistance of their haemagglutinin. The fluids still showed haemagglutinating power after being heated to 62° for 30 min. Their other characters are described in the following section.

Table 2. *Isolation of virus from mice at zone of partial interference*

(The day of death or the day on which it was killed (K) is shown after each mouse. The dilutions are those of supernatant fluid from a 10 % suspension of mouse brain in saline.)

Virus mixture in original inoculum	Mouse	Virus isolated at dilutions			
		10^{0}	10^{-1}	10^{-2}	10^{-3}
NWS 10^{-3} + WSM 10^{-1}	69,738 K7	NM, N′, N′, N	.	− − −	.
	69,739 +9	NM, N′, +	.	+, −	.
	69,740 +9	N′, N′, N′	.	− − −	.
NWS 10^{-3} + WSM 10^{-2}	70,450 +5	+ + −	NM, N, +	NM, NM − −	− − −
	70,451 +6	N, − − −	+ − − −	− − − −	.
	70,452 K7	NM, N, + −	+ − − −	− − − −	.
	70,453 K7	N, − −	N, − −	− − −	.

NM, N′, N = virus present of the type indicated; + = virus present but not further investigated, usually because of low titre; − = no virus present.

Isolation and characterization of NM strains

The striking features of the NM strains were their isolation from mouse brain and hence by implication their encephalitogenic capacity, and the heat resistance of their haemagglutinin. These appeared to correspond to qualities specific for the two original strains and immediately raised the question as to whether a recombination of the qualities of the two viruses had occurred. Obviously the first requirement was to ensure that fluids showing these qualities did not contain a mixture of virus types. The only way in which certain characteristics of an infective fluid can be shown to be the manifestation of a single clone of virus is to obtain secondary clones by subinoculation at limiting dilutions. If all the positive fluids obtained have the same qualities as each other and as the parent fluid, the likelihood of single clones being concerned becomes very high. In the present instance it is necessary to show that repeated re-isolation at limit dilution from infective fluids or mouse-brain emulsions regularly gives fluids which are infective for mice by intracerebral inoculation, and which show haemagglutination after being heated to 62° for 30 min. The evidence in this respect for the first NM strain isolated is as follows. The first positive fluid 51,557 from mouse-brain 69,738 showed haemagglutination on the 3rd and not on the 2nd day of incubation. It therefore may well have been infected with only a single particle. Mouse-brain 70,138 infected from 10^{-2} dilution of this fluid was titrated in chick embryos giving egg fluid 52,901 at the limit infective dilution of 10^{-3}. This fluid titrated in embryos gave infective fluids including 53,243 at 10^{-7} and not at 10^{-8}; it killed mice on intracerebral injection at 10^{-5}. Table 3 shows the relevant characteristics of the three fluids described above, together with a parallel series derived from mouse-brain 70,139 a duplicate of 70,138.

Table 3. *Characters of fluids containing NM type of virus*

	Haemagglutinin			
			Encephalitis	
Fluid	Original	After heating at 62° for 30 min.	Dilution	*
51,557	200	130	10^{-2}	(6), (6), 7, 7
52,901	320	140	10^{-5}	8, 8, 8, 8
53,243	420	240	10^{-2}	6, 6, 6, 8
52,910	240	120	10^{-4}	9, 9, 9, 11
			10^{-5}	7, 8, 9, S
53,251	180	100	10^{-2}	5, 6, 8, S

* Note as at foot of Table 1.

The same general procedure has been followed in isolating the other strains. Four distinct isolations have been made from mice receiving mixtures of NWS and WSM, those concerned (all shown in Table 1) having the numbers I 69,738, II 69,739, III 70,450, IV 70,452. Strain I is that shown in Table 3.

Strains I and IV were highly pathogenic for mice by the intracerebral route but II and III much less so, giving a proportion of survivors from test inoculations of allantoic fluids diluted 10^{-1} or 10^{-2}. Of sixteen mice inoculated intracerebrally with one or other of these dilutions of fluids derived by limiting-dilution passage from strain II, four died at 6, 6, 7, 8 days, four were killed for culture when sick at 6, 7, 8, 8 days, and eight survived. All such fluids contained haemagglutinin which resisted heating for 30 min. at 62°. The other three NM strains, all of which had undergone two or more limit-dilution transfers to the allantoic cavity, were tested in parallel for the main characters that can be determined by *in vitro* tests.

Position in the receptor gradient. The position in the receptor gradient was determined by the use of fowl cells treated with graded dilutions of receptor-destroying enzyme (RDE; Burnet, 1951*a*). Table 4 shows the results with three NM strains and the other significant viruses, as well as the results of tests made on fowl cells treated with each of the WS types and stabilized with anti-serum. These cells were treated in the proportion of 0·1 ml. of packed fowl cells to 5 ml. of undiluted allantoic fluid virus for 4 hr. at 37°. It will be seen that NM strains are considerably further along the gradient series than any of the other WS types. They produce full agglutination of cells rendered inagglutinable by the homologous virus through the action of NWS or WSM. In this character they are by no means intermediate between NWS and WSM and it is obvious that here no question of the NM strains being mixtures can arise.

Table 4. *Position of NM strains in receptor gradient*

	Cells treated with RDE dilutions						Cells treated with		
Strain	1/50	1/100	1/200	1/400	1/800	1/1600	WSM	NWS	NM-WS
WSM	−	−	−	+ ±	+ +	+ +	−	+	−
NWS 54,530	−	−	−	±	+ +	+ +	−	−	−
NM 54,208 I	−	+	+ +	+ +	+ +	+ +	+ +	+ +	−
NM 54,222 III	−	+	+ +	+ +	+ +	+ +	·	·	·
NM 54,238 IV	−	±	+ +	+ +	+ +	+ +	·	·	·
NDV	−	−	−	±	+ ±	+ +	·	·	·
MEL	−	−	−	±	+ +	+ +	·	·	·
WSE	−	−	±	+ +	+ +	+ +	·	·	·
LEE	±	+ +	+ +	+ +	+ +	+ +	·	·	·

+ + = complete agglutination; ± ±, +, ± = degrees of partial agglutination; − = no agglutination of cells treated as shown and tested with five agglutinating doses of virus.

Serological character. There are minor serological differences between NWS and WSM in the sense that a ferret anti-serum against NWS has a considerably higher titre against the homologous strain than against WSM. A WSM anti-serum on the other hand neutralizes both strains to approximately the same extent. Comparative tests of three NM strains, NWS and WSM against ferret sera 304 NWS and 305 WSM are shown in Table 5. The NM strains are inactivated a little less readily than WSM and are relatively insensitive to the action of anti-NWS ferret serum. A similar relative insusceptibility was evident with two immune fowl sera (anti-NWS).

Tests for neutralization *in ovo* (allantoic method) were limited to the experiment whose results are shown in Table 6. This confirms the fact that serologically the NM strains are closer to WSM than to NWS.

Table 5. *Neutralization of viruses (anti-haemagglutinin) by ferret anti-sera*

(Figures give anti-haemagglutinin titres, i.e. the dilution of serum which mixed with five agglutinating doses of virus allows partial (+) agglutination of fowl red cells.)

Strains		F 304 (NWS)	F 305 (WSM)
WSM	54,322	250	1000
NWS	54,314	1000	2400
NM I	54,208	150	1000
NM III	54,222	100	600
NM IV	54,238	150	800

Table 6. *Neutralization of NM strain III* (54,222) in ovo *by ferret anti-sera*

(Mixtures contained $10^{6 \cdot 8}$ 50 % infective doses of virus.)

V 1/5 plus	F 304 (NWS)	F 305 (WSM)
S/10	+ + + +	+ + + +
S/3	+ + + +	+ + − −
S undiluted	+ + + +	− − − −

V = virus; S = serum.
+ = infection as indicated by haemagglutination; − = no infection.

In mice that have been immunized with NWS by combined intranasal and intraperitoneal inoculation of formalin-killed virus, intracerebral inoculation of NM virus produces no signs. It is noticeable (Table 7) that strain III is less virulent than the other two, in this respect resembling strain II which was not included in this experiment.

Table 7. *Resistance of mice immunized with NWS against intracerebral challenge with NM strains*

Strain		Dilution	Immune mice*	Control mice of same age
NWS	54,026	10^{-2}	S, S, S, S	6, 6, 7, 7
NM I	54,197	10^{-2}	S, S, S	8, 8, 8, 8
NM III	54,222	10^{-2}	S, S, S, S	8, 12, S, S
NM IV	54,238	10^{-2}	S, S, S	6, 7, 7

* Note as at foot of Table 1.

Enzymic activities. The NM strains can readily be converted into indicators of the inhibitory effect of ovomucin on haemagglutination. They show about the same degree of activity in destroying ovomucin inhibitor as WSM; i.e. much more than NWS. In limited experiments no qualitative differences from other WS strains could be observed either as indicator or enzyme. The NM strains resemble WSM and NWS, and differ from WSE in being capable of stabilizing red cells treated with excess of virus at 37° without the necessity for subsequent treatment with immune serum.

Heat resistance. The comparative heat resistance of the various types which have been mentioned is shown in Table 8. The fluids were all freshly harvested stocks and were heated, undiluted, for 30 min. at the temperatures shown. The close correspondence of the heat resistance of NM to WSM is obvious.

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Table 8. *Heat resistance of haemagglutinins of WS subtypes*

(Figures give haemagglutinin titre after fluids had been heated for 30 min. at the temperature shown.)

Temperature of heating (° C.)	Virus type			
	NWS	N′-WS	NM III	WSM
Unheated	320	480	480	640
54	<2	100	320	640
56	.	<2	320	600
60	.	.	280	450
64	.	.	120	360
67	.	.	<10	<10

DISCUSSION

The evidence shows that from mixed infections with the strains NWS and WSM a new type can be isolated with some regularity in which the thermostable haemagglutinin of WSM and the encephalitogenic quality of NWS are both manifested. The capacity to produce encephalitis is more marked in some strains than in others, but two *in vitro* characters are shown by all, the heat resistance of the haemagglutinin already mentioned and a quality shown by neither ‘parent’, in the form of a sharply changed position in the receptor gradient. Before the appearance of these strains can be ascribed to recombination it must be established that the fluids whose characters have been tested do in fact consist of pure clones of virus, or more correctly that they do not represent mixtures of the two original strains. We consider that a simple examination of the qualities shown in Tables 4, 5 and 7 will show that no hypothesis of mixture is tenable. A large number of other fluids obtained at various stages in the process of isolation, mouse passage and re-isolation from limit dilutions was similarly tested with the same *in vitro* findings. This uniformity of character rather than any formal proof from the fact that each strain had undergone two or more passages at limiting infective dilution, is the most important indication that a new virus type is being dealt with.

The NM strains are unlike any strain in previous use in this laboratory and the possibility of casual contamination can be excluded. Since they resemble both WSM and NWS serologically and fail to infect mice that have been immunized with NWS, the possibility can be eliminated that some unsuspected neurotropic virus derived from the mice is being activated by the intracerebral injections.

In the circumstances the only possibilities that can be considered are that the NM strains are derived by mutation from one or other strain, or that the new strain is the result of some type of fusion of genetic qualities from both the original types.

All the virus forms with which we have been concerned in this paper are descendants of the first strain of human influenza virus isolated by Andrewes, Laidlaw and Smith. In one sense therefore NM must be regarded as a mutant irrespective of the process by which it was produced, and it may be formally impossible to exclude its origin by direct mutation. We have definitely adopted the working hypothesis that recombination of characters has in fact occurred, but it would be unjustifiable to speculate further as to the mechanism involved until a completely unequivocal example of recombination can be provided.

Further work has shown that similar recombinant strains can be obtained from mice inoculated with NWS and influenza virus A of different serological subtype. In these strains encephalitogenic activity is combined with the serological character of the non-neurotropic original strain. A more complete discussion of the problems presented by these phenomena will therefore be deferred until these strains are described.

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A Genetic Approach to Variation in Influenza Viruses

4. Recombination of Characters Between the Influenza Virus A Strain NWS and Strains of Different Serological Subtypes

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SUMMARY: Mice were inoculated intracerebrally with mixtures of the influenza virus strain NWS and the serologically distinct non-neurotropic strains MEL, OcI and SW. From mouse brains removed about the 5th or 6th day virus strains were obtained which, after at least two passages at limiting infective dilution, showed active encephalitogenic power with the serological character of the second strain of the original mixture. These are regarded as recombinants. The strains in general show *in vitro* characters close to those of the serologically similar 'parent'. They have, however, diminished enzymic action on ovomucin and do not readily elute from red cells; in this they resemble the strain NWS. They are more readily converted to the indicator state than either of the original strains.

The process by which these unusual types arise is discussed in the light of recent views that intracellular multiplication of bacterial viruses and of some animal viruses takes place not by binary fission but by the breaking down of the infective unit into smaller particles which are the replicating units. It is concluded that the recombinants arise by reconstitution of infective particles from the pool of replicating units produced by double infection of a single cell by virus particles of both parent types.

In a previous paper (Burnet & Lind, 1951) we described the isolation from the brains of mice infected intracerebrally with mixtures of the strains NWS and WSM, of four strains which had characters apparently derived from both 'parents'. It was of obvious interest to see whether similar recombination and characters could be obtained by this method, using strains which, as well as having no encephalitic action, were serologically distinct from NWS.

In this paper we report the production of several strains in which serological characters corresponding to MEL, SW and OcI were combined with encephalitogenic action. In this way we have now available recombinant strains of each of the major serological subtypes of influenza A virus resulting from their interaction with the strain NWS.

MATERIALS AND METHODS

The methods used were described by Burnet (1951 *a*, *b*), Burnet & Lind (1951) in earlier papers of this series. The only important modification used in our later experiments was to emulsify brains from mice infected with mixtures of NWS and another virus in a 10% solution in saline of serum from a fowl immunized against NWS. In this way it was possible to inhibit, not always completely, growth of any NWS that might be present when the brain emulsions were tested in chick embryos.

Virus strains

The NWS strain used was described by Burnet (1951*a*).

MEL. The Melbourne strain adapted to mouse passage during 1935–6, but since 1940 maintained by storage of amniotic or allantoic fluid at the temperature of solid CO_2. For the experiments from which Nr-MEL II, III and IV were derived a 'pure clone' stock of MEL was used.

SW. Shope's strain swine 15 received through Dr C. H. Andrewes in 1936, and maintained as was MEL.

OcI. The strain isolated from the Ocean Island epidemic of 1948 by Isaacs, Edney, Donnelley & Ingram (1949). This is antigenically of the CAM-FMI (A^1) group and shows the characteristic filamentous morphology under dark-ground examination. After isolation by amniotic inoculation in chick embryos it was adapted to allantoic passage, re-isolated at limit infective dilution and maintained as glycerolated seed virus.

EXPERIMENTAL

There are two main sets of experiments. The first set deals with the origin and characters of the first two strains which were regarded as recombinants of NWS with MEL and SW respectively; the second set with experiments carried out in 1950, essentially to establish the reproducibility of the phenomena. In these anti-NWS serum was used to assist the recovery of recombinants in the presence of NWS and the procedure thereby greatly simplified. Both the strains to be described in the first section of the paper were derived in a single experiment designed primarily to observe the range of viruses which on simultaneous injection would interfere with the encephalitogenic action of NWS.

NWS fluid 54,026 was diluted 10^{-3} and 0·25 ml. volumes were mixed with equal amounts of undiluted allantoic fluid viruses as shown in Table 1. In the case of MEL, dilutions of 10^{-1}, 10^{-2}, 10^{-3} were also included, with a control mixture in which an extra volume of the diluent, normal horse serum saline (10 % serum), was used. The viruses in this experiment were stock fluids harvested from 0 to 6 days previously.

Each mixture was inoculated intracerebrally into four mice (4–5 weeks old) in 0·03 ml. amounts. The mice were observed for 12 days. A large proportion of those showing signs of encephalitis or dying later than the control mice were examined for amount and type of virus present in the brain. The whole brain was removed aseptically and ground with coarse alundum powder before being emulsified in 2 ml. physiological saline. The suspension was spun lightly and the supernatant fluid inoculated at dilutions from undiluted to 10^{-3} in 11-day chick embryos. All positive fluids at or near the limiting infective dilution were tested for serological type with immune ferret sera. Those which showed evidence of serological character other than WS were then studied in detail.

The results are shown in Table 1 from which it will be seen that interference was complete with the two WS strains and PR8, and virtually complete with the other A strains, MEL and BEL. With SW there was some evidence of interference, none with LEE, Mumps or NDV. Most of the strains isolated

Table 1. *Interference with encephalitogenic action in mice of NWS by other viruses*

Added virus	Deaths	Virus isolations in eggs from mouse brains indicated by A, B or C
MEL undiluted	(8)A, – – –	A – – –
MEL diluted 10^{-1}	5A, 5B, 6C, 8	A: N, N, N; B: N, N, MEL; C: N, + +
Mumps	5, 6, 6, –	.
NDV	5, 5, 5, 6	.
WSM	– – – –	.
WSE	– – – –	.
BEL	(8)A, – – –	A: N, N
PR8	+ – – –	.
SW	5A, (7)B, 7C, –	A: N, SW; B: N, N, N; C: N, N
LEE	5A, 5B, 7C, 7	A: N, N; B: N, N; C: N, N, N
Control	5, 6, 6, 6	.

5, etc. = death on 5th day after injection; (8) = mouse sick and killed on 7th day, which would probably have died on 8th day; – = survival for 12 days.
N = virus resembling NWS serologically.
MEL, SW = virus showing this serological character present in the allantoic fluid.
+ = virus present but not further characterized, usually because of low titre of fluid.

from brain emulsions were of the NWS type. Some may have been of N′ type (Burnet & Lind, 1951) but this was not studied. The two strains indicated as MEL and SW had the serological character of the corresponding non-neurotropic strain in the mixture used. Details of the titration of the mouse brains are shown in Table 2.

Table 2. *Titration of mouse brains from which recombinants were obtained*

Mouse no.	Virus added	Virus isolated in eggs			
			Dilution		
		Undiluted	10^{-1}	10^{-2}	10^{-3}
70,555	MEL	+ + –	N, N, +	MEL, – –	– – –
70,594	SW	+ + +	SW, N, –	– – –	.

Symbols as in Table 1.

Purification and characterization of the strain Neuro-Swine Nr-SW I

The allantoic fluid shown in Tables 1 and 2 as of SW serological character (fluid from egg 54,598 inoculated with mouse-brain 70,594) was inoculated undiluted into the brains of four mice. Five days later one was dead and two were very sick. From the brains of these three animals virus of the same SW serological type was isolated. One fluid of this group, (54,914) whose characters are shown in Table 3, was titrated in eggs, and the fluids from the limiting infective dilution of 10^{-8} tested. All positive fluids showed the typical serological reactions of a swine strain and one of these (55,198) tested in mice at a dilution of 10^{-3} produced typical symptoms in all, with death in the two not killed for culture. Emulsions of these two brains were infective for chick embryos up to a dilution of 10^{-3}, and allantoic fluids from this dilution were of Swine serological type. All the allantoic fluids that have been mentioned had similar characteristics, and we refer to the type of virus involved as Neuro-Swine (Nr-SW) this particular strain being Nr-SW I.

The sequence of passages from which the stock glycerolated seed virus of Nr-SW I was derived can be represented as follows:

$$\text{Mixture NWS}+\text{SW}\rightarrow\text{M}\rightarrow\text{E}\rightarrow\text{M}\rightarrow\text{E}\xrightarrow{\text{L}}\text{E (55,198)},$$

where M is mouse inoculation, E is allantoic inoculation and L is passage at the limiting dilution.

The characters of pathogenicity for mice by intracerebral injection and neutralization in anti-haemagglutinin tests by Swine antisera are shown in Table 3.

Table 3. *Serological character and pathogenicity of Nr-SW strain*

(Titres shown represent dilution of ferret serum neutralizing five agglutinating doses of virus in anti-haemagglutinin tests.)

		'Antisera'				
	Passage	NWS 304	SW 303	MEL 310	LEE 309	Intracerebral pathogenicity for mice
E 54,598	0	10	100	15	<10	10^0 5, (6), 6
E 57,914	2	15	120	.	.	10^4 (7), 7, 7, 8
E 55,198	3	<50	240	.	.	$10^{-2\cdot2}$ (7), (8), 8, 8
SW	.	20	320	<20	<20	10^0 — — —

The capacity to produce encephalitis is well marked, mice dying when inoculated with fluids diluted 10^{-4} and their brains yielding relatively large amounts of virus. Brain emulsions were usually infective for eggs up to a dilution of 10^{-3}. The stock SW strain from which the recombinant was derived had no influence on mice inoculated intracerebrally either with undiluted or 10^{-2} allantoic fluid virus. In the only experiment so far completed interference with the encephalitogenic action of Nr-SW I is strongly shown by MEL and WSM and partially by LEE. Mixtures of Nr-SW I diluted 10^{-3} with equal volumes of undiluted virus gave no deaths with MEL and WSM, three delayed deaths and one survival with LEE.

Purification of the strain Nr-MEL I

The original fluid from eggs inoculated with mouse-brain emulsion was heavily contaminated with bacteria but had a high enough haemagglutinin titre to allow its characterization as a MEL-type strain. This was treated with penicillin and diluted 10^{-8} for passage. Two positive and two negative fluids were obtained, but both positive fluids were still contaminated. We then applied the simple procedure of shaking the fluid with toluene and after leaving for 1·5 hr. at 37° inoculating eggs with a 10^{-2} dilution, These gave virus-containing fluids free from bacteria. One of these fluids, 55,014, was inoculated into mice undiluted and also diluted 10^{-2}. One of four mice inoculated with the undiluted material (M70,738) died on the 6th day, the other three survived. In the group inoculated with 10^{-2} dilution of virus there was also only one death (M 70,734), in this case delayed until the 12th day. From mouse 70,738 two eggs inoculated with brain emulsion diluted 10^{-1} gave positive fluids, both with MEL serological character. A further allantoic passage at limiting infective dilution 10^{-9} gave fluid 55,841. On testing this in mice intracerebrally the virus was much more pathogenic than in the first passage, giving deaths on the days indicated as follows: undiluted, 3, (5), 7; diluted 10^{-2}, (6), 7, 7. Brains from one mouse in each group showed virus to a dilution of 10^{-3} of MEL serological type. Stock virus Nr-MEL I is derived from a further limit-dilution passage of virus derived from this series, its history being expressible as follows:

$$\text{mixture MEL}+\text{NWS} \rightarrow \text{M} \rightarrow \text{E} \xrightarrow{\text{L}} \text{E} \rightarrow \text{M} \rightarrow \text{E} \xrightarrow{\text{L}} \text{E} \rightarrow \text{M} \rightarrow \text{E} \xrightarrow{\text{L}} \text{E} \text{ stock 56,608.}$$

The *in vitro* characters of Nr-MEL described in the next section were assessed on fluids from eggs inoculated with the glycerolated stock fluid 56,608.

In vitro *characters of Nr-SW and Nr-MEL strains*

It is obviously of the greatest interest to find whether the development of neurotropism is regularly associated with any other demonstrable change in the virus. The strains Nr-SW I and Nr-MEL I were therefore examined in parallel with the normal strains and NWS for most of the measurable *in vitro* characters of influenza viruses. All tests were made with allantoic fluid virus not more than 5 days old, obtained by inoculating 11-day embryos with 10^{-6} dilution of glycerolated seed virus and incubating at 35° for 2 days.

The thermostability of the haemagglutinin of the neuro-strains was almost identical with that of the normal types. The result of heating virus buffered with phosphate to pH 7·35 for 30 min. at temperatures between 52 and 60° is shown in Table 4. There is no significant change in position in the receptor gradient. Using the standard method with fowl cells treated with graded amounts of RDE the results shown in Table 5 were obtained. When attempts were made to compare the reactions of cells which had been treated directly by the two viruses a clear distinction between Nr-SW and SW was found. After 5 hr. action of excess virus at 37° cells treated with SW were found to be stable and to have lost their agglutinability by all the available viruses. Cells treated with Nr-SW, on the other hand, showed no sign of stabilization and they could

Table 4. *Thermostability of haemagglutinin from original and recombinant strains*

(Figures show haemagglutinin titre given by allantoic fluid virus heated for 30 min. at the temperature shown.)

		Temperature				
	Titre of unheated strain	52°	54°	56°	58°	60°
Virus strain		Heamagglutinin titre				
MEL	480	·	420	480	360	25
Nr-MEL I	300	·	240	280	110	30
SW	200	160	70	<2	·	·
Nr-SW I	250	80	50	<2	·	·
NWS	320	·	<2	·	·	·

be stabilized only by the use of an appropriate dilution of anti-SW ferret serum. When tested the only virus failing to agglutinate these cells was NDV. Both SW and Nr-SW were fully active. The deduction that Nr-SW was adsorbed to, but had little or no enzymic action on, the receptor substance of the cell surface was confirmed by measuring directly the adsorption and elution of the viruses to fowl cells. Both were adsorbed well but after 5 hr. at 37° the supernatant fluids showed titres of SW 160, Nr-SW 2, compared with original haemagglutinin titres of 500 in both instances.

Table 5. *Place in receptor gradient with fowl cells trated with graded dilutions of receptor-destroying enzyme (RDE)*

(Symbols show the relative degrees of agglutination by five agglutinating doses of virus, of cells treated for 30 min. with dilutions of stock enzyme (titre 1/1500.))

Virus strain	RDE dilutions					
	1/25	1/50	1/100	1/200	1/400	1/800
	Degree of agglutination					
MEL	−	−	−	±	++	++
Nr-MEL I	−	−	−	+	++	++
SW	−	±	++	++	++	++
Nr-SW I	−	+	++	++	++	++
NWS	−	−	−	−	−	++

An almost identical relationship exists between Nr-MEL and MEL. In one experiment the two stock viruses were diluted so that each had a haemagglutinin titre of 250 and 0·125 ml. of packed fowl cells added in the cold to 4 ml. of virus. After 20 min. at 0° the tubes were spun and the supernatant fluid removed. This was replaced by 4 ml. of saline and the resuspended cells placed in a water-bath at 37°. At the end of 2 hr. it was obvious that the cells treated with MEL were relatively stable while those treated with Nr-MEL were grossly unstable; this persisted when the tubes were removed after 4 hr. at 37°. After separation of the supernatant fluid the cells were washed (after stabilization with immune serum in the case of Nr-MEL cells) and tested for their susceptibility to agglutination by different viruses; the results are recorded in Table 6. The cell reactions are unusual in that Nr-MEL leaves NDV and MEL receptors apparently intact while greatly diminishing its own reactivity; MEL, on the other hand, removes NDV and MEL receptors but has no greater action on Nr-MEL receptors than the homologous virus.

Both new strains are more readily converted to the indicator state than the originals. Nr-MEL is of special interest in being convertible to indicator by simple heating for 30 min. at 56° which, as Stone (1949) has shown, is without effect on the standard virus. In the presence of citrate at pH 8·5 heating to 50° converts Nr-MEL to a sensitive indicator while MEL requires a temperature between 52 and 54°.

The SW and Nr-SW strains are much more readily converted to the indicator state. Simple heating at 50° brings out the full susceptibility of Nr-SW I but has only a minimal effect on most samples of SW. Occasionally older fluids of

Table 6. *Comparison of action of MEL and Nr-MEL on fowl cells*

(The upper half of the table shows the differences between MEL and Nr-MEL when absorbed to fowl cells in the cold and then allowed to elute at 37°. The figures show the haemagglutinin titres of supernatant fluids. Nr-MEL is adsorbed but not eluted. The second half of the table shows the reactions of cells treated with MEL or Nr-MEL and stabilized when tested with five agglutinating doses of the six viruses shown.)

	Virus	
	MEL	Nr-MEL
	Haemagglutinin titre	
Fluids		
First supernatant 0°	2	22
Supernatant 2 hr. at 37°	280	16
Supernatant 4 hr. at 37°	300	10

	Testing virus					
	NDV	NWS	WSM	Nr-MEL	MEL	LEE
Cells treated with virus	Reaction of cells					
Nr-MEL	++	−	−	+	++	++
MEL	−	−	−	+	−	++

Table 7. *Conversion of normal and 'neuro' types of MEL and SW to the indicator state*

(Figures shown are dilutions of ovomucin solution giving end-point inhibition of haemagglutination by five agglutinating doses of virus. For all instances not otherwise shown, heating was for 30 min.)

	MEL	Nr-MEL
	Dilution of ovomucin	
Treatment		
Heated 56°	<10	3,000
Heated with citrate at pH 8·5		
50·5°	20	30,000
52°	2,000	·
54°	30,000	30,000
56°	30,000	·
	SW	Nr-SW
Heated 50°	100	<10,000
Heated with citrate at pH 8·5		
50°	10,000	20,000
37° (2 hr.)	300	20,000

Nr-SW have been found which showed indicator activity without being heated (see Table 7).

Enzymic action on ovomucin. Both neuro-variants are much less active than the standard strains in destroying the inhibitor quality of ovomucin. As pointed out in previous papers the extent to which inhibitor, especially inhibitor for the indicator-treated MEL (T-MEL) (Stone, 1949), is destroyed, often depends greatly on the concentration at which the ovomucin is present in the reacting mixture. With many samples of ovomucin, MEL fails to act unless the ovomucin is diluted to a level equivalent to about 2000–3000 inhibitor units.

With the sample used in the present experiments both MEL and SW completely destroyed inhibitors at the highest concentration of ovomucin used. The method described by Burnet (1949) was used, a final dilution of twenty agglutinating units of virus being present in a final volume of 1·25 ml. Ovomucin prepared according to the method of Gottschalk & Lind (1949) was diluted 1/5 in Ca saline lightly buffered with acetate at pH 6·2, then centrifuged. The supernatant fluid was used in 1 ml. vol. and 0·25 ml. of each virus, adjusted to a strength of 100 agglutinating units was added. A drop of toluene served as a bacteriostatic substance and the corked tubes were held for 18 hr. at 37°. Residual haemagglutinin was eliminated by heating to 62·5° for 30 min. The mixtures were then titrated for inhibitor using six indicator viruses WSE, LEE, MEL, Nr-MEL, SW and Nr-SW, each treated in the fashion appropriate to the strain to give a fully sensitive indicator. The results are recorded in Table 8.

Table 8. *Action of viruses on the inhibitor qualities of ovomucin*

(Figures show dilution to which five agglutinating doses of indicator virus are inhibited.)

Ovomucin acted on by	Indicator strain					
	H-WSE	H-LEE	T-SW	T-Nr-SW	T-MEL	T-Nr-MEL
Nil	3,000	3,000	8,000	8,000	30,000	20,000
MEL	<10	<10	<10	<10	<10	<10
Nr-MEL	100	80	250	250	8,000	6,000
SW	<10	<10	<10	<10	<10	<10
Nr-SW I	80	80	250	80	30,000	12,000

With ovomucin initially diluted 10^{-1} there is a much less evident difference. Both Nr-MEL and Nr-SW destroy all inhibitor for T-MEL or T-Nr-MEL under these conditions, but Nr-MEL leaves a small residual inhibition against heated WSE (H-WSE) and T-SW.

Isolation of further strains of Nr-MEL and Nr-SW

It seemed desirable to make a fairly elaborate study of the first two strains before attempting to obtain further recombinants of the same type. In two more experiments on similar lines we had no difficulty in obtaining several more strains. It will have been noted that the neuro-strains studied can be distinguished from normal strains by a simple *in vitro* procedure. Nr-MEL strains heated to 56° for 30 min. take on indicator status, whereas MEL strains do not. The same difference between Nr-SW and SW strains is found when the temperature is 50°. If these differences are typical then any infected fluid obtained by subculture from mouse brain can be rapidly characterized by testing it serologically against the two appropriate ferret sera and, after heating, for indicator status against ovomucin dilutions. With one exception discussed below there has been general concordance of mouse pathogenicity by intracerebral route with these *in vitro* characters. Another refinement of technique has been to add anti-NWS serum (made by immunization of fowls) in a final concentration of 1/10 to all dilutions of mouse-brain emulsions, and to hold the dilutions thus prepared at 4° for 1 hr. before inoculating into eggs. Thus we can prevent the appearance of the original non-neurotopic form, since it cannot survive for 5–6 days in the brain and by means of the anti-NWS serum we can prevent growth of the original NWS unless there is very active multiplication.

The procedure was standardized along the following lines. Mice were inoculated with mixtures containing NWS diluted 10^{-3} and equal volumes of the non-neurotropic virus fluid, undiluted or diluted 10^{-1} and 10^{-2}. In all these experiments the seed viruses used had been prepared from 'pure clone' fluids. At the 5th or 6th day mice with the smallest proportion of the second virus were usually dead or dying and at this stage mice with minimal symptoms in the other groups were killed for culture. The brains were removed, ground with saline containing 10 % of serum from fowls 285 or 286 (anti-NWS) and 100 units penicillin/ml. After centrifugation and a variable period in the refrigerator the supernatant fluid was inoculated undiluted or diluted 10^{-1}, 10^{-2}, 10^{-3} in horse-serum saline containing anti-NWS serum 1/10 into 11-day embryos by the allantoic route. After 3 days all positive fluids were tested for neutralization of haemagglutinin by ferret antisera corresponding to the two strains in the mixture. All strains serologically NWS were discarded. Those neutralized by the other serum were then tested for conversion to the indicator state by simple heating, and a representative example from the highest infective dilution of brain substance chosen for passage at limiting dilution. Dilutions of 10^{-7}, 10^{-8}, 10^{-9} were subinoculated to 11-day embryos and the limit positive fluid tested similarly *in vitro* and then inoculated diluted 10^{-1} into mice intracerebrally. From a sick or dead mouse at 5 or 6 days virus was re-isolated by the same procedure and a further limit-dilution passage made. This final fluid was then tested for its power to produce encephalitis when injected intracerebrally at 10^{-1} dilution and its *in vitro* characteristics checked.

The history of the strain Nr-SW III was as follows:

$$\text{NWS } 10^{-3}+\text{SW } 10^{0}\to \text{M}_1\to \text{E}_1 \xrightarrow{\text{L}} \text{E}_2\to \text{M}_2 \xrightarrow{\text{L}} \text{E}_3 \xrightarrow{\text{L}} \text{E}_4\to \text{M}_3$$

In Tables 9 and 10 is shown the character of the mixture inoculated and the primary egg fluids obtained. The first-limit egg passage corresponds to fluid E_2 in the schema and the second to E_4. For each is shown the number of the fluid used, the dilution of the previous fluid that infected the embryo in question, and the death-times of mice inoculated intracerebrally with fluid diluted 10^{-1}. The Nr-SW strains were uniform in character, indistinguishable from Nr-SW I and approximately equally pathogenic for mice. Similarly, Nr-MEL III and IV closely resemble Nr-MEL I and no further discussion of these is required.

The results with one mouse-brain emulsion from an animal, M 70,903, inoculated with MEL+NWS were however extremely complex and a variety of types was obtained. These results may be highly relevant for an understanding of the process of recombination and we therefore give them in some detail. In the first egg culture of the brain emulsion the 10^{-1} dilution gave four positive fluids all serologically MEL; but two showed the Nr-MEL character of conversion to indicator by simple heating at 56° (57,210, 57,212) and two failed to do so (57,209, 57,211). Application of the usual procedure to 57,212 gave the results shown in Table 11. The first limit-passage fluid produced sickness but

Table 9. *Isolation of recombinants from mixed infections with NWS and SW*

Strain	From mouse no.	Inoculum	Primary fluids	First-limit passage	Second-limit passage
Nr-SW II	70,914	NWS dil. 10^{-3}+SW dil. 10^{0}	10^{0}: SW, SW, SW 10^{-1}: SW*, SW, SW	10^{-8} 57,505, 5, 6, 6	.
Nr-SW III	70,915	NWS dil. 10^{-3}+SW dil. 10^{0}	10^{0}: N, N, N, SW* 10^{-1}: N, N, – –	10^{-8} 58,437, 5, 5, 5	10^{-8} 59,186, 5, 5, 5, 6
Nr-SW IV	71,026	NWS dil. 10^{-3}+SW dil. 10^{0}	10^{0}: N, SW, SW, + 10^{-1}: N, N, SW*, +	10^{-8} 58,091, 5, 5, 6, (6)	10^{-8} 58,915, 3, 4, 8, 9
Nr-SW V	71,028	.	10^{0}: N, – – – 10^{-1}: SW*, N, – – –	10^{-7} 58,339, 5, 5, 5, 5	10^{-8} 59,173, 4, 6, 6

N = strain with serological character of NWS; SW = strain with serological character of SW.
* Fluids used for further passage. All fluids shown had serological character of SW.
Days of death of mice inoculated with 10^{-2} fluid are shown after each.

Table 10. *Isolation of recombinants from mixed infections with NWS and MEL*

Strain	From mouse no.	Inoculum	Primary fluids	First-limit passage	Second-limit passage
Nr-MEL II	70,903	NWS dil. 10^{-3}+MEL dil. 10^{0}	10^{0}: M, M, M, M, M 10^{-1}: M, M, M, M*	A 10^{-9} 58,586, (6), (6), S, S B 10^{-9} 58,450, 3, 5, 5, (6)	10^{-8} 59,152, S, S, S, S 10^{-7} 59,360
Nr-MEL III	71,016	NWS dil. 10^{-3}+MEL dil. 10^{0}	10^{0}: M, M, M, M 10^{-1}: M, M, M, M*	10^{-9} 58,384, 3, 3, 4, 4	10^{-9} 59,062, 5, 5, 5, 6
Nr-MEL IV	71,017	NWS dil. 10^{-3}+MEL dil. 10^{0}	10^{0}: M, M, M, – 10^{-1}: M, M, M*, –	10^{-9} 58,397, 5, (6, 6, 6)	10^{-9} 59,217, 5, 6

M = fluid with serological character of MEL; S = survived for 12 days.
Other symbols as Table 9.

did not kill mice intracerebrally. There was, however, virus to a dilution of 10^{-2} in the brain of one of the sick mice. A further limit passage from egg to egg gave fluid 59,152 which failed to kill any of four mice, although all showed minor symptoms around the 6th day. All fluids in this series were of Nr-MEL type *in vitro*.

Fluid 57,209, of normal MEL character, was injected intracerebrally into three mice. One, killed when sick at 5 days, gave fluids all of Nr-MEL character by *in vitro* test. The second, killed when moribund at the 6th day, gave two types of fluid in approximately equal numbers, one typified by 58,255 had the *in vitro* qualities of Nr-MEL. The other typified by 58,260 was not significantly neutralized by either MEL, NWS or SW antisera and was convertible into indicator by simple heating. This strain has maintained its character on limit-dilution passage and is reserved for future study. Fluid 58,255 of Nr-MEL character was passed at limit dilution and killed four mice at 3, 5, 5 and (6) days. Nr-MEL type virus was isolated from the brains and after another limit-dilution transfer was inoculated to mice intracerebrally. These died at 5, 6, 6, (7) days.

Table 11. *Character of different substrains obtained from 'mouse' 70,903*

(All strains were of the serological type MEL.)

Representative fluid	Indicator heated at 56°	Intracerebral pathogenicity for mice	
58,255	+	3, 5, 5, (6)	
57,508	+	3, 6, (6), (7), S, S	
57,579	+	S, S, S, S	Mice appeared sick around 6th day
58,586	+	S, S, S, S	
58,574	±	S, S, S, Non-pathogenic	
57,582	−	S, S, S, Non-pathogenic	

+ = fluid converted to indicator for inhibition by ovomucin and giving full titre; ± = indicator but titre low. 5, etc. = death on 5th day after injection; (6), etc. = mouse sick and killed 7th day, would probably have died on 8th day; S = survived for 12 days.

Several other lines of descent were followed from mouse-brain 70,903 and a summary of those series in which at least one limit-dilution fluid was tested is given in Table 11. The interest of this series lies in the strong indication of a graded series of strains of increasing intracerebral pathogenicity. If we are concerned with recombination of characters there is here a possible opportunity of analysing what appears to be a 'dosage effect'. As soon as circumstances allow it is hoped to return to this aspect of the problem.

Isolation of a recombinant of NWS with a filamentous influenza A^1 strain

In the hope that an additional genetic 'marker' could be obtained in the filamentous character of recent influenza A^1 strains, limited experiments were made with the strain, Ocean Island (OcI.) (Isaacs *et al.* 1950). The procedure was exactly as with MEL and SW and three lines were followed. The results resembled in many respects those obtained with MEL in the appearance of three more or less distinct subtypes.

The most encephalitogenic substrain Nr-OcI. II was obtained from fluid 58,215 derived from 10^{-1} dilution of mouse brain 71,119 inoculated with (NWS diluted 10^{-3}+OcI. diluted 10^{-1}). Fluid 58,491 obtained by limit-dilution passage (dilution 10^{-7}) of 58,215 was diluted 10^{-1} and inoculated intracerebrally into mice one of which died on the 7th day; the others were killed and cultured at 5, 5 and 7 days respectively, all appearing sick at the time. The brain emulsion from one was infective for eggs to dilution 10^{-3}, the fluids having OcI. serological character and showing no filamentous forms on dark-ground microscopy. Fluid 59,211 representing a second limit passage (dilution 10^{-9}) of 58,215 gave, on intracerebral inoculation in mice, one death at 2 days, while the three remaining mice were all very sick on the 7th day but recovered subsequently.

Another strain Nr-OcI. I, similarly derived from mouse 71,118, gave throughout OcI. serological reactions and showed definite but non-fatal illness in mice inoculated with first limit-dilution egg-passage fluid. The sick mouse killed on the 5th day gave a brain emulsion infecting two of four chick embryos at dilution 10^{-3}. A further limit-dilution passage gave at dilution 10^{-7} the fluid 59,189. In mice this fluid produced sickness at 6–8 days but all survived. Our impression was that the symptoms of excitability and undue disorientation after being spun by the tail and dropped back into the tin, were less marked than in mice inoculated with the first limit-passage egg fluid.

The third series was started with fluid 58,189 from a 10^{-2} dilution of brain emulsion of mouse M 71,083 killed when sick at 5 days. This fluid was serologically OcI. and, unlike the other fluids mentioned above, contained an almost normal proportion of filamentous forms. None of the mice inoculated became sick, and eggs inoculated from the brain of one killed at 5 days were not infected. Apart from the fact of its survival for 5 days in the first mouse of the series there is nothing to indicate any difference from normal OcI. The association of long filaments with non-neurotropic forms and their almost complete disappearance in the encephalitogenic Nr-OcI. II is a point of considerable interest which it is hoped to investigate more fully.

DISCUSSION

We have taken the obvious viewpoint that when two viruses are inoculated into a mouse brain, one neurotropic of serological character A, the other non-neurotropic of serological character B, and a neurotropic strain serologically B is obtained, then a recombination of characters has occurred. The fact that a similar phenomenon has been described for bacterial viruses (Hershey & Rotman, 1948; Delbrück & Bailey, 1946) makes it likely that an important general character of viruses is concerned and justifies a certain amount of speculation as to the mechanism by which recombination of characters occurs.

It must be admitted that no direct information is available on the morphological changes undergone by viruses in the course of multiplication in the cell. Electron micrographs have not shown any unequivocal evidence of partially completed binary fission in the purified suspensions needed for electron microscope work. There is, however, no decisive evidence against the view that the standard method of reproduction by animal viruses is by simple binary fission although there is undoubtedly a strong trend of present opinion away from this view. Hypotheses as to the basis of the recombination phenomena described in these papers must therefore be shaped according to whether reproduction of influenza viruses is assumed to be by binary fission or by some alternative method.

A. The available hypotheses on the assumption of binary fission appear to be three in number:

(1) Under certain circumstances two virus particles in the same cell may undergo a primitive type of sexual fusion allowing some redistribution of characters when the 'haploid' form is regained. This would be analogous to the situation which appears to obtain in regard to the formation of bacterial recombinants with the *E. coli* strain K 12 (Tatum & Lederberg, 1947).

(2) Mutation is relatively frequent in viruses, and it is possible that in the course of intracellular multiplication non-viable mutants are abundantly produced. By analogy with the work of Avery *et al.* (1944) on pneumococcal type transformation, one could conceive that a unit analogous to the deoxyribosenucleic acid of the pneumococcus could be transferred from one type of virus to a mutant deficient in the corresponding allele, thereby rendering it viable and giving opportunity for recombination of characters.

(3) It is possible that a strain adapted to growth in a special intracellular environment such as brain, may in some way facilitate the rapid transformation of another virus into a form capable of growth in the unusual environment. There is perhaps a remote analogy in the phenomenon of syntrophism in bacteria when two variants lacking capacity to synthesize two different essential metabolites may be able to grow in mixed culture in a medium unable to support growth of either alone. We know of no closer analogy.

B. If we follow the current trend of opinion amongst workers with bacterial viruses that the coliphages T_2, T_4 and T_6 at least multiply by a method totally distinct from binary fission, a second general hypothesis can be formulated.

Workers with bacterial viruses of this group consider that after the virus particle penetrates the bacterial surface it breaks up into 30–50 sub-units (genes). These replicate independently and towards the end of the phase of intracellular development, infective virus particles are reconstituted ready for liberation with lysis of the host cell. The evidence is based first on Luria's (1947) work on the infectivity of virus treated with ultra-violet light which indicates that while one virus particle is non-infective a high proportion of multiple infections give rise to multiplication and lysis. In addition, there is the extensive evidence of recombination provided by Hershey & Rotman (1948) and by Delbrück & Bailey (1946).

Hoyle (1948), from his work on the disappearance of influenza virus for a few hours after infection of the allantoic lining, and Bauer (1949), from a general consideration of the problem of virus multiplication, have already suggested that similar processes are concerned in the intracellular growth of animal viruses. Hoyle considers that the disappearance of demonstrable virus in the allantoic membrane is due to a disintegration of the infective particles into a similar non-infective form. The existence of this form can be demonstrated by the presence of the 'soluble' complement-fixing antigen in the tissues before the reappearance of infective and haemagglutinating virus. Whether this overall identification of the 'non-infective phase' of the virus with the complement-fixing antigen is justifiable may be doubtful but the temporary disappearance of demonstrable virus has been confirmed by Henle & Henle (1949) and for inactive (indicator) virus by Isaacs & Edney (1951) and must in some way be accounted for.

Obviously our results could readily be covered by a slight elaboration of Hoyle's hypothesis along the lines that have been adopted by Delbrück (1949) to interpret the genetics of bacterial viruses. One would have to assume not that the virus disintegrated into a soluble phase composed of uniform particles, but that the units produced were of the nature of individual genetic units replicating true to type and that the equivalent units in related but distinct virus strains had allelomorphic relations to one another.

In the Nr-OcI. strain derived from recombination of NWS and OcI. it has been shown that *two* characters of NWS have been transferred to the recombinant, neurotropism and spherical morphology. We have taken the serological type of the recombinant as the most fundamental character and have usually thought of the recombinants as representing the non-neurotropic form on to which the neurotropic character has been in some way grafted. However, considering the relatively small number of markers that are available, our results are quite compatible with the view that the process envisaged by Hoyle does take place and allows wide possibilities of recombination of characters. The strains isolated are of course only those combinations capable of survival and growth in the conditions provided by brain tissue and the manipulations of isolation in the allantoic cavity.

It should be stressed that any genetic work with influenza viruses must be done within the strict technical limitation that there is no equivalent to a plating method by which all the offspring of a given 'cross' can be determined and no possibility of isolating single cells in which double infections have been initiated. The technique must always be to introduce the two parent strains in relatively high concentration into the susceptible tissue and then so to arrange circumstances that the multiplication of both original types is inhibited relative to that of the recombinant one is seeking. The obvious procedure is to take strains which differ (*a*) serologically, (*b*) in ability to multiply in a given tissue. The strains are now placed in an environment in which one only can multiply and at an appropriate stage anti-serum to inhibit the activity of this strain is used; only recombinants should then appear, as in the experiments we have detailed. But any such method can obviously only allow the isolation of strains with the capacity to overcome both the obstacles provided. If recombination were a straightforward sexual interchange of qualities AB × A′B′, giving the possible recombinants AB′ and A′B, only one of these could be isolated. With our mouse-brain technique and NWS as a constant component, for example, it is impossible to obtain as a recombinant non-neurotropic WS.

There are already hints from the experiments with MEL that more than one type of recombinant can be obtained and further work in this direction should be illuminating. We feel that our results require the acceptance of some process allowing free recombination of characters either by sexual fusion of two individuals or by the diffuse replication of individual 'genes' according to Hoyle's hypothesis. Without by any means feeling that the last word has been said we rather strongly favour the latter alternative.

Many workers have been impressed by the general resemblance between the larger bacterial viruses and influenza viruses, and the evidence in regard to bacteriophage multiplication and recombination is extremely cogent. When all the demonstrable characters of influenza virus multiplication and recombination agree with such an hypothesis it is natural to lay much stress on the analogy, although technical considerations make it impossible to devise with influenza virus the fully quantitative studies possible with bacteriophages. The well-established temporary disappearance of demonstrable influenza virus in the early stages of infection is important evidence against binary fission as the standard process of multiplication. The existence of filamentous forms of virus (Chu, Dawson & Elford, 1949) in recent influenza A strains, in itself seems to favour strongly a method of growth in which the final infective form is built up from a pool of self-replicated sub-units. With such a method it is easy to conceive of the production of a variety of infective units from spheres to long filaments. It is not easy to see how filaments could be produced by any process resembling binary fission.

We feel that our findings add strong circumstantial evidence in favour of the hypothesis that influenza virus particles are composite, and on entry into the host cell break down into sub-units which replicate independently, giving rise to a pool of virus material from which infective units can be reconstituted. This hypothesis has not been established, but it has implications which could be tested. (1) The initial stage of virus disintegration soon after entering the cell is presumably due to a relatively simple enzyme action and should be capable of imitation *in vitro*. Disintegration should be demonstrable by the disappearance of infectivity and haemagglutination and by electron microscope studies. (2) A simple accumulation of data on the types of recombinant obtained by the experimental technique we have been using should provide indication of how far the recombination process is a symmetrical one. (3) There seems to be no intrinsic reason why classical genetic procedures such as backcrossing should not be modified to the circumstances and provide valuable information at the genetic level. (4) With advanced optical and electron microscope techniques the fate of filamentous virus in tissue culture might be followed and provide direct evidence of breakdown and reconstitution.

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The Conversion of Certain Soluble Sugars to a Glucosan by Holotrich Ciliates in the Rumen of Sheep

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SUMMARY: When *in vitro* fermentations of glucose, fructose or sucrose are carried out under conditions comparable with those in the rumen by means of relatively small inocula of strained rumen contents from hay-fed sheep, the holotrichously ciliated protozoa which are present store immense numbers of microscopic granules consisting of a practically protein-free glucosan giving a purple colour with iodine. The granules can be liberated by bursting the protozoa by the action of the synthetic detergent Teepol XL under mild conditions. An exactly similar polysaccharide granule preparation can be made from protozoa present in the rumen itself if rumen contents are withdrawn 2–4 hr. after feeding. The yield of iodophilic polysaccharide so obtained may be equivalent to approximately 25% of the weight of water-soluble, yeast-fermentable sugars in a single feed of hay. No granule-filled protozoa are present 9 hr. after feeding.

Other simple sugars such as glucuronolactone, galactose, mannose, xylose, arabinose, sorbose, lactose, cellobiose and maltose, are not markedly converted into polysaccharide granules by rumen protozoa, during *in vitro* fermentation.

The most obvious group of micro-organisms ordinarily inhabiting the rumen of sheep are the large ciliated protozoa. Bulk for bulk, although of course not numerically, they usually exceed the bacterial population (Mangold, 1929). Nevertheless, they appear not to be entirely essential for the adequate functioning of at least some rumen fermentations, since defaunated sheep have been shown to continue to thrive (van der Wath & Myburgh, 1941; Johnson, Hamilton, Robinson & Garey, 1944). The current view is therefore, not unnaturally, that the protozoa, from the point of view of fermentation reactions, are of minor importance when compared with the rumen bacteria (see Elsden & Phillipson, 1948). A very simple experiment (recorded later) having disproved this extreme view *in one particular instance*, namely glucose fermentation by the rumen contents of hay-fed sheep, it became of interest to find out the action of rumen protozoa on fermentable sugars. No claim is here made that protozoa are important in all rumen fermentations. This paper is mainly concerned with the storage of polysaccharide granules during carbohydrate fermentation by one important group of protozoa—the holotrichously ciliated—usually present in the sheep's rumen.

METHODS

Animals. Rumen contents were obtained as required from two Suffolk-cross ewes, Sheep 272 and 337, aged 2½ years, fitted with permanent rumen fistulae by Dr J. Tosic (1949). They had been fed exclusively on good-quality starch-free meadow hay, containing about 14% of water-soluble constituents of which about one-seventh was yeast-fermentable carbohydrate (probably fructose), for several months before the experiments. Their weights had been well maintained during this period. Each was fed at 12 hr. intervals with 800–900 g. hay; nearly all the food was consumed within 1 hr. Sheep 272 was chiefly used but 337 gave similar results whenever its rumen contents were used in parallel experiments.

In vitro *fermentation of simple sugars by rumen contents.* The method was essentially that of Elsden (1946) for glucose, with small Buchner flasks as fermentation vessels; in order to quicken the fermentation the inoculum of strained rumen-liquor was usually increased from 10 to 17 % (v/v), an extra buffering agent in the form of sodium acetate (0·5 %) was added (this is permissible because rumen contents always contain this salt), and the saturation with CO_2 from an external source was omitted. The same end was achieved more quickly by adding the requisite amount of glacial acetic acid, last of all to the buffer mixture containing bicarbonate, arranging matters so that after the addition of the inoculum the Buchner fermentation flask was full to the side-arm. The final concentration of sugar was always 0·9 % or less and the pH value at the beginning of the fermentation was 6·8–7·0. The fermentation flasks were shaken from time to time.

This simple fermentation system was used to demonstrate that the true fermentative action of protozoa was on occasion at least of the same order of magnitude as that of rumen bacteria. Muslin-strained rumen contents (500 ml.; see below) taken 3 hr. after feeding was allowed to stand for 1 hr. at 40° in a tall beaker covered with a clock glass. The supernatant liquor was then carefully decanted from the bottom protozoan layer, the latter was washed twice by decantation with the Elsden buffer (50 ml.) to remove most of the free bacteria, and then made up to 20 ml. Fermentations with 10 ml. of the final protozoan suspension and 30 ml. of the first rumen supernatant (rich in bacteria but still containing some protozoa) were run side by side, each fermentative liquid containing initially 0·4 % glucose. The pH 7 buffer used in each case to make up the reaction volume to 100 ml. was prepared with sodium carbonate (not bicarbonate) and acetic acid; the medium was then not supersaturated with CO_2 at the start. The gases evolved during the fermentations were collected over warm water. After 4 hr. at 40° the protozoan fermentation had evolved two and a half times as much gas (22 ml.) as the (mainly) bacterial fermentation (9 ml.). It must be remembered that the protozoan fermentation was due to the large organisms from some 250 ml. of rumen liquor, as against 30 ml. for the bacterial fermentation; on the other hand, the so-called 'bacterial' fermentation was partly due to small protozoa which did not sediment readily. There was, in fact, a distinct layer of protozoa on the bottom after 4 hr.

Separation of the larger protozoa from rumen contents or from in vitro *fermentation liquids.* The method of straining through six layers of surgical gauze without squeezing allowed surprisingly little plant debris through into the filtrate, but in order to collect the greater part of the larger protozoa it was necessary to scrape the deposit from the gauze, resuspend it in distilled water and re-strain through clean gauze. The combined filtrates were then stood for at least 1 hr. in boiling-tubes filled to the top, when the larger, whiter, and heavier protozoa gradually sank to the bottom. The supernatant was carefully decanted, the deposit washed several times by decantation with 0·003 M phosphate buffer (pH 6·8) and finally with distilled water. The final deposits contained relatively very few free bacteria and consisted almost entirely of protozoa together with a very little plant material. As will be seen from the results reported, many fermentation liquids contained polysaccharide-filled protozoa which were very easily and quickly washed clean by the above simple method, because they settled rapidly under the influence of gravity, being much more dense than their unfilled fellows or than water or rumen liquor itself.

Liberation of the granules from washed protozoa. This was readily achieved with the holotrichs by incubation at 39° for 1–2 hr., with occasional shaking, in about one-tenth of the original rumen liquor volume of a solution of Teepol XL (0·2 %) in 0·003 M phosphate buffer (pH 6·8). (Teepol XL; British Drug Houses Ltd. is sold as a powder giving an almost neutral solution in water and is said to be the sodium salt of a secondary alkyl sulphate.) The tube was then allowed to stand at room temperature for 1–2 hr. until most of the protozoan debris and unburst protozoa of other genera had settled to the bottom as a greyish deposit below a milky supernatant liquor. The latter, which on examination with the high dry objective was seen to consist almost entirely of a suspension of tiny granules, was carefully decanted and the granules therein collected by centrifuging. The deposit was twice resuspended in water, separated by decantation and finally collected and dried *in vacuo* over concentrated H_2SO_4 at 39° for several days. The dried preparations were analysed for total nitrogen (micro-Kjeldahl) and for reducing sugar by Neish's (1946) modification of the Shaffer-Somogyi method after hydrolysis with 0·55 N-HCl at 100° for 2 hr., followed by neutralization with N-NaOH. In certain instances the alkaline iodine method of Hinton & Macara (1924), under conditions applicable to aldose sugar only, was also used. No attempt was made to determine the carbohydrate content of preparations containing more than 2 % N.

RESULTS

Storage of iodophilic granules in protozoa during in vitro *fermentations with a small inoculum of rumen liquor*

The only simple sugars with which a visible fermentation began within 1–3 hr., when the inoculum was derived from rumen liquor in its most active state (viz. 2–4 hr. after feeding of the sheep), were glucose, fructose, sucrose, galactose and xylose. With arabinose, mannose, cellobiose, lactose, maltose, glucuronolactone and soluble starch, there was a longer delay before gas evolution was observed. With sorbose, or with no added sugar at all, no obvious fermentation took place. In the case of the first three sugars listed a conspicuous, pure white, sandy layer of protozoa had settled to the bottom of the fermentation vessel within 3 hr., and this heavy white layer persisted apparently unchanged even after 24 hr. provided that the initial sugar concentration was not too great. It could then easily be collected, washed by decantation, and thus separated from a much less dense and more flocculent greyish green layer situated above it. The bottom white layer consisted mostly of large holotrichs, together with some equally large oligotrichously ciliated protozoa (*Diplodinium*, etc.). Every one stained solidly brownish purple with iodine. The more flocculent greyish green layer contained representatives of both groups, sometimes individually iodophilic, together with clumps of bacteria and some plant debris. A similar bottom layer was obtained with the other fermentable sugars, but iodophilic protozoa were not especially in evidence therein, and indeed, with xylose, almost entirely absent. The action of Teepol XL upon the heavy white protozoan bottom layer was to cause nearly all the iodophilic holotrichs to burst, thereby liberating an uncountable number of discrete granules from each ruptured organism. These granules stained intensely purple-brown with iodine; leaving the protozoan cases and macro-nuclei, often distorted in shape, stained only a yellowish brown. The granules varied in size from that of large cocci to small yeasts, and were of an assortment of shapes, chiefly elliptical or roughly circular. A few much larger pseudo-crystalline particles were also seen, usually five or six-sided. These were apparently not derived from the holotrich ciliates but from the rare bursting of smaller oligotrich ciliates. If a wet preparation was made by mixing one drop of the milky granule suspension with one drop of Loeffler's methylene blue, the protozoan debris took the stain whereas the granules did not. Likewise, if a heat-fixed preparation was stained, the granules were found not to take any of the ordinary bacterial stains.

The inference that the granules were almost entirely polysaccharide in nature was confirmed by analysis of the various dried preparations made from *in vitro* fermentations. The results are shown in Table 1 which also includes some analyses carried out on the protozoan granule preparations made from strained rumen liquor itself, taken 2–3 hr. after feeding of the sheep. The purification by washing of the iodophilic protozoa was somewhat more difficult in these latter experiments, but relatively protein-free granule preparations could nevertheless be obtained. The results in Table 1 further show clearly that a synthesis of protozoan polysaccharide granules must have taken place in the *in vitro* sugar fermentations. This conclusion was supported by a comparative microscopic examination of iodine-stained wet preparations, when it was seen that the washed protozoa from *in vitro* fermentations were on the average much whiter, larger and stained more solidly, than those present in the rumen-liquor used as inoculum.

Influence of 'age' of inoculum on the in vitro *synthesis of polysaccharide granules*

By 'age' of inoculum is meant here the time in hours between the feeding of the sheep and the withdrawal of the rumen-liquor through the fistula. Sheep 272 was used for an experiment in which the various 'ages' were 2, 4, 6·5, and 9 hr. Fermentations were carried out in each instance upon fructose *in vitro* using the customary 17 % (v/v) inoculum of strained rumen-liquor. After 24 hr. all the fermentation liquors contained a copious white bottom layer of iodophilic protozoa which was easily washed, by decantation, with distilled water. Holotrichs predominated in all save in that from the 9 hr. inoculum where the iodophilic oligotrichs perhaps predominated. The nitrogen content of

Table 1. *Analysis of dried protozoan preparations obtained from rumen liquor and sugar fermentations therewith*

(All results are calculated for an inoculum of 100 ml. of rumen liquor; fermentation period with sugars, 20 hr.)

		Protozoan debris after action of Teepol XL		Polysaccharide granule preparation by action of Teepol XL			
						Reducing sugar after hydrolysis, as glucose	
Sugar fermented (mg.)	Inoculum (%, v/v)	Weight (mg.)	N content (%)	Weight (mg.)	N content (%)	By Shaffer-Somogyi (%)	By Hinton-Macara (%)
None. (Rumen liquor 2 hr. after feeding sheep 337*)	—	124	6·0	18	0·40	86	—
None. (Rumen liquor 2 hr. after feeding sheep 272)	—	54	7·5	41	1·84	—	—
None. (Rumen liquor 3 hr. after feeding sheep 272†)	—	17	3·0	43	0·39	77	80
As above, on another day	—	—	—	34	1·55	—	—
Glucose: 5300	17	—	—	125 (1·4% ash)	0·14	91	—
Fructose: 5300	17	—	—	164	0·18	90	90
Sucrose: 1330	86	—	—	100	1·96	78	77
Glucose: 10,000	10	—	—	110	0·135	—	—

* Yielded 220 mg. of dried whole protozoa with nitrogen, 5·1 %.

† This specimen of rumen liquor gave a much whiter bottom protozoan deposit than usual.

the respective dried preparations of whole protozoa so obtained are given in Table 2. It will be noted that the last preparation listed, which as mentioned above contained relatively more iodophilic oligotrichs than usual, had a much higher nitrogen content than the others. Attempts to burst the oligotrichs at a neutral pH by means of Teepol XL were unsuccessful.

Table 2. *Nitrogen contents of dried whole protozoa obtained after fructose fermentation with small inocula of strained rumen liquor of various ages*

		Washed and dried protozoa/100 ml. of rumen-liquor inoculum	
Time elapsing between feeding of the sheep and removal of inoculum (hr.)	Type of protozoon: (H = holotrich, O = oligotrich)	Weight (mg.)	N content (%)
2	H	50	1·61
4	H	60	1·44
6·5	H	73	1·13
9	H, O	27	3·51

The immediate working-up of samples of the original inocula for polysaccharide granule preparations gave results which by no means paralleled the above. Only the first two, for instance, gave heavy white bottom layers on standing; the last (9 hr.) inoculum gave hardly any iodophilic protozoa at all, and the relatively insignificant granule preparation made from the greyish green deposit yielded by it consisted chiefly of non-iodophilic bacteria, as its nitrogen content likewise showed. These results are summarized in Table 3. It will be seen that only the granule preparations from the 2 and 4 hr. inocula contained less than 2 % nitrogen. Microscopic examination showed that at 6 hr. after feeding the iodophilic protozoa in the rumen had clearly lost the greater part of their polysaccharide granules, and that these had entirely disappeared after 9 hr.; yet, as Table 2 shows, the holotrich protozoa themselves were still capable to some extent of resynthesizing polysaccharide granules *in vitro* if presented with a fermentable sugar, e.g. fructose.

Table 3. *Nitrogen contents of protozoan granule preparations obtained from rumen liquor* (100 *ml.*) *of various ages*

Time between feeding of the sheep and removal of rumen liquor for protozoa isolation (hr.)	Type of 'granule' (P=polysaccharide, B=bacteria)	Washed and dried granule preparations	
		Weight (mg.)	N content (%)
2	P	19	1·01
4	P	32	1·74
6·5	P, B	18	3·73
9	B	8	5·84

Properties of the granule preparation

The dried granule preparations of low nitrogen content (see Table 1) could easily be resuspended in water to give a milky liquid which, when gradually heated, cleared at 70–80° owing to the bursting of the granules. The now faintly turbid liquid was kept at 100° for 5 min., cooled, tested for reducing sugars (absent) and for bacteria by means of a wet preparation made with Loeffler's methylene blue (only a very few bacteria visible). It gave a purple colour with iodine reminiscent of that given by amylopectin, but quite unlike that usually given by glycogen. The chemical structure of this polysaccharide is now being investigated by Prof. E. L. Hirst and Dr E. G. V. Percival at Edinburgh.

There can be little doubt that the main simple sugar formed on hydrolysis, even when the preparation is derived from a fructose fermentation, is essentially glucose, since the reducing-sugar by alkaline-iodine oxidation was always equal to that determined by copper reduction (see Table 1). No hydrolysate gave the Seliwanoff reaction for fructose. Phenylglucosazone (m.p. and mixed m.p. 204°) was in one instance prepared from the hydrolysate of granules obtained from a fructose fermentation.

DISCUSSION

Although the holotrich ciliates (e.g. *Isotricha* and *Dasytricha*) are not usually regarded as the most abundant type of protozoon in the rumen of sheep (Mangold (1929) gives about 10 % of the total numbers for representatives of this group, as against 40 % each for the oligotrichs *Diplodinium* and *Entodinium*) they are clearly the most active in depositing polysaccharide granules inside themselves when glucose, fructose or sucrose is present. The process is so rapid, and the extent of polysaccharide synthesis so considerable, that there can be little doubt that the protozoa themselves directly convert these simple sugars into iodophilic polysaccharide granules. It seems probable that a considerable fraction of the water-soluble and yeast-fermentable carbohydrate (presumably fructose) in the hay fed to the sheep in these experiments is stored in this form, at least temporarily, in rumen protozoa. A feed of 800 g. hay would contain roughly 15 g. of simple yeast-fermentable sugars. Assuming a total rumen-liquor content of 10 l. and that average samples are in fact withdrawn through the fistula, it can be calculated, from the figures given in Table 1 for sugar fermentations, that 2–3 hr. after feeding, as much as 4 g. at least of protozoan polysaccharide granules have been synthesized and stored in holotrich ciliates alone. Owing to inevitable experimental losses the real amount stored must be considerably greater than this, even if no allowance be made for the lesser but obviously not inappreciable amount synthesized from simple sugars by oligotrich ciliates such as *Diplodinium* and *Entodinium*.

These granules cannot be micro-organisms since they contain far too little protein (less than 1 % in the best preparation) and far too much carbohydrate (up to 90 %). Buchanan & Fulmer (1928) listed eighteen species and strains of anaerobic and facultatively anaerobic bacteria with average nitrogen content of 9·5 % (limits, 4·0–14·2 %), and pointed out that even yeasts rarely contain more than 60 % of carbohydrate in their dried bodies. The synthesis of iodophilic polysaccharides by micro-organisms of the digestive tract is of course a well-known phenomenon, studied, for example, by Nasr & Baker (1949) who, however, pointed out that this process is incompletely understood, particularly as concerns yeasts and protozoa.

The storage of protozoan polysaccharide granules in the rumen is only temporary since no trace of them could be obtained on working-up protozoa 9 hr. after feeding the sheep. It remains to be shown whether protozoa auto-ferment their polysaccharide store, or whether bacteria play a part in this process.

Of the three yeast-fermentable hexoses having a common enolic form, namely glucose, fructose and mannose, only the first two seem to be utilized *in vitro* by the holotrichous protozoa for making polysaccharide granules. An analogous example was observed by Kluyver & Hoppenbrouwer (1931) who showed that *Termobacterium mobile* Lindner did not ferment mannose, although it readily fermented glucose and fructose, and, more slowly, sucrose. The fact that the glucose-containing disaccharides maltose and cellobiose are apparently not readily utilized by *Isotricha* and other holotrichs for iodophilic polysaccharide synthesis shows further how strictly limited are their synthetical powers.

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New Techniques for the Study of Growing Micro-organisms

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SUMMARY: A method is described for the examination of growing micro-organisms by vertical and oblique incident illumination using an illuminator of the 'Universal Illuminator' (Messrs Cooke, Troughton and Simms) type. A moist chamber has been developed to enable this method to be used for the routine examination of bacterial cultures, and examples are given of both aerobic and anaerobic cultures on the surface of solid nutrient media, preferably containing nigrosine.

This method has also been used for following spore germination of *Bacillus subtilis* in a liquid medium. A method using Cellophane as a support for growth is also described. Certain organisms can be grown on Cellophane 'windows' made in the caps of Bijou bottles containing a liquid medium; this apparatus is suitable for the repeated but not continuous examination of young growths.

The methods commonly used for the continuous observation of growing micro-organisms under the microscope suffer from the defects of difficulty or inconvenience in manipulation, and often of unnatural restraint upon the organisms themselves. In this paper we describe the application of two systems of illumination which have long been current among metallurgists for observations on opaque specimens, viz. 'vertical' and 'oblique incident' illumination. Even in biological work these are far from novel. Thus the 'Lieberkühn' is a simple form of oblique illuminator; it is 200 years old, and was originally conceived by Descartes (Carpenter, 1891). While pleas for their more extended use have appeared from time to time (e.g. Vonwiller, 1927) they have not in our opinion been applied to the extent that they deserve. Our object therefore is to suggest that the efficient and convenient modern form of apparatus might well become a routine tool in microbiological work, not largely confined, as seems to have been so far, to occasional highly specialized investigations.

The optical equipment is described briefly, partly because of its unfamiliarity to some workers, partly because its practical form will be seen to conduce greatly to the ease of manipulation which can be attained. Apart from a description of general features, we consider in detail a number of applications; examples are chosen principally to indicate the range that can be covered though each has some point of intrinsic interest.

Vertical and oblique incident illumination

In vertical illumination, light from an illuminated iris, brought nearly parallel by means of a lens, is directed towards a thin glass plate lying on the axis of the microscope immediately behind the objective. The plate is tilted so as to reflect part of the light directly downwards through the objective, which then forms an image of the iris on the surface of the specimen under observation.

If the specimen reflects specularly, only a brightly illuminated circle is seen; irregularities of contour appear dark wherever the surface departs sufficiently from normality to the axis, or scatters some of the incident light.

Oblique incident illumination is the same as ordinary dark ground, except that the hollow cone of light is applied from above instead of below the specimen. A parallel hollow cylindrical beam is formed by an annular diaphragm in front of the lamp, and reflected by an annular mirror in the axis of the microscope; the down-going beam surrounds the light path from the objective to the eye. It is focused by a bispherical condenser as in the usual dark-ground system. The objective is specially constructed in a narrow tube passing through a central hole in the condenser and supported by a spider. A more detailed description can be found in, for instance, Langeron (1948).

The image formed by the one method of illumination is, roughly speaking, the negative in the photographic sense of that formed by the other. In both cases 'metallurgical' objectives—computed for use without a cover-glass—are used.

Vertical illumination is normally capable of giving information only about the surface of a specimen; the change of refractive index on passing from air to specimen is large, and so most of the light reaching the eyepiece is that which has been reflected at the surface. Optical inhomogeneities within a transparent object are seen with difficulty, if at all; they usually reflect less light than an air-solid interface, and that little is lost in the much brighter reflexion from the surface.

Oblique illumination is capable of reaching objects embedded in transparent solid or liquid media, since the ground remains dark. The useful depth is limited by the deterioration in image quality that is tolerable. With a 4 mm. objective, about 50μ. is the maximum.

We have throughout used a microscope designed for metallurgical work, i.e. one with a stage which can be raised and lowered by rack and pinion, which will accommodate large objects, and whose whole top surface can be moved in both directions at right angles to the microscope axis. Further, although the illuminators can be fitted with self-contained lamps, an external and more powerful source is better; once this has been aligned with the mirror, the coarse adjustment of the body cannot afterwards be moved, and stage focusing is a necessity. Most of our work has been carried out with the 'Universal Illuminator' (Messrs Cooke, Troughton and Simms) which embodies both optical systems in one fitting. A common mirror is used, its centre being clear and its outer part silvered. Two tubes carrying the necessary stops and lens are mounted side by side on a slide attached to the body; by interposing one or the other between lamp and mirror either a central or an annular beam is admitted. Thus either method of illumination can at once be brought into play by a simple manual adjustment. The objective need not be changed, and the dark-ground condenser remains in position.

This arrangement has two great advantages. First, the rapid interchange of vertical and oblique illumination makes it possible to obtain without refocusing and searching all the information available from a given object under these conditions. Secondly, focusing itself is much facilitated when only dark-ground observations are required; it is carried out as follows. The vertical illuminator is brought into position and its field iris nearly closed. The specimen is then raised towards the objective until the image of the iris is seen sharply defined on its surface. This occurs suddenly and cannot easily be missed. On then switching over to dark ground it is certain that the surface is still in focus though the particular field may be optically empty. There is thus no danger of damage to the front lens of the objective, or of contamination from live pathogenic organisms in the specimen.

The apertures of the objectives used with oblique incident illumination are limited by the fact that part of the solid angle above the specimen must be occupied by the illuminating beam. The highest dry power available is a 4 mm., N.A. 0·65, initial magnification 45. With vertical illumination there is no such inherent limitation; there is provision in the 'Universal Illuminator' for replacing the dark-ground condenser and special objectives by objectives of normal construction, which may be of the highest power and aperture.

The special virtue of these forms of illumination is thus that they facilitate direct examination of micro-organisms growing on, or at a short distance below, the surfaces of liquid or solid media. There is little restriction on the form—especially the depth—of the container. For following the growth of a micro-organism which is left in position under the microscope, we prefer to work in a room held at a constant temperature of 35°. The physiological discomfort is less irksome than the restriction on speed and accuracy of manipulation imposed by a 'hot box', and the absence of temperature gradients confers many advantages. A heat filter between lamp and specimen is essential in work with growing organisms; without it they are rapidly killed. A poor filter allows fogging of the objective by vapour rising from the medium. We have found a 6 mm. total thickness of Chance's ON 19 heat-absorbing glass to be entirely satisfactory.

Examination of growth on solid media

Media for oblique incident illumination. When the oblique incident illuminator is used on the surface of agar, the image seen suffers from lack of contrast owing to Tyndall scattering in the gel, which produces a fairly bright field. Further, ordinary media are never free from foreign bodies, crystals, or globules of fat; these introduce confusing and irrelevant detail. The difficulty can be overcome by adding to the medium a dye which absorbs light strongly (cf. Klieneberger & Smiles, 1942); for this purpose we have used nigrosine. A 10% (w/v) stock solution of nigrosine in water is Seitz-filtered after adjustment of the pH to neutrality. One volume of the solution is added to 10–20 vol. of molten medium. Though it is convenient to treat fairly large amounts of routine media in this way, we use a more economical method with special media of which perhaps only one or two plates are required occasionally. A small quantity of a common medium such as tryptic meat agar, with nigrosine, is melted, and a thin layer poured over the surface of the special medium; the plate is then allowed to stand for 24 hr. or more, when sufficient diffusion will have taken place for the uppermost surface to have acquired the particular properties of the underlying medium. Plates made from media prepared in this way are extremely opaque; foreign bodies and other inclusions give little trouble since those at a depth of more than a few microns are not illuminated. It is important, however, that the surface should be as free from blemish as possible. Usually it is sufficient to pour the medium at 60° or greater; this allows a considerable time for settling before it solidifies. The common impurities in the surface itself, notably collections of fat globules, become familiar by experience, and for many purposes can be disregarded. Where there is likely to be confusion, we pass the whole medium while hot through a Seitz filter before pouring, thus obtaining plates whose surfaces are almost entirely featureless over large areas.

Tests have been made of the ability of organisms to grow on media containing nigrosine. The test species ranged in general robustness from *Bacillus subtilis* to *Neisseria meningitidis*, and in complexity of food requirements from *Azotobacter chroöcoccum* to *Haemophilus pertussis*. Plates of untreated medium and of the medium with added nigrosine were inoculated in pairs and incubated. The results are shown in Table 1. With only three organisms was the growth obviously restricted namely, *Brucella suis*, *Br. tularensis*, and *Pfeifferella mallei*—and in these only where the inoculum was sparse. One other result deserves notice. The growth of some organisms is inhibited by small quantities of gentian violet. Our *Staphylococcus aureus* failed to grow on a medium containing 0·001 % of the dye, but with added nigrosine growth proceeded normally. Nigrosine combines with triphenylmethane dyes (Knaysi, 1944), and it seems that it thereby neutralizes their bacteriostatic properties.

Since nigrosine is usually available in bacteriological laboratories, and because it proved to be so widely applicable as a light absorbent, we made no extended tests of other materials. It should not be difficult to find substitutes in cases where its presence is objectionable.

Media for vertical illumination. With vertical illumination, no special treatment of the bulk medium is called for, since only the surface itself can be seen. Foreign particles and corrugations in the surface must of course be avoided. Organisms which grow in or upon the surface of a medium are best examined with vertical illumination; in oblique light they produce very intense scattering and flare, and details are obscured.

Observations of growth on solid media

It is a very simple matter to watch the growth of a young colony of micro-organisms on a nigrosine-nutrient agar plate in the conditions described above at a magnification of 400–600 diameters. For the best results, however, attention must be paid to the size of the inoculum and to the dryness of the medium. Evaporation from the latter causes recession of the surface (which must be avoided during photographic exposures), a hardening of the surface layers through concentration of the agar, and a consequent progressive change in the appearance of bacterial growths. Some form of moist chamber is therefore desirable. Our apparatus is described in the following account of our general procedure, using *Streptococcus lactis* as an example.

Table 1. *Growth of organisms on media containing nigrosine*

Organism	Medium	Growth: Without nigrosine	Growth: With nigrosine incorporated	Growth: With nigrosine layer
A. chroöcoccum (freshly isolated from soil)	PMM	+	+	.
	TMA	+	+	.
B. anthracis (4 fully virulent and 1 avirulent strain)	**CCY**	+	+	.
	TMA	+	+	.
B. licheniformis	**TMA**	+	+	.
	GL	+	+	.
	PA	+	+	.
Bacillus group 9 species other than *anthracis* and *licheniformis*	**TMA**	+	+	.
	PA	+	+	.
Bact. coli	**TMA**	+	+	.
	MacConkey's	+	.	+
	Desoxycholate	(+)	.	(+)
Br. suis (a fully virulent strain)	TMA	+	(+)	.
	Liver extract agar	+	.	(+)
Br. tularensis (a fully virulent strain)	TMA	(+)	(+)	.
	Francis's*	+	.	(+)
Chr. prodigiosum	**TMA**	+	+	.
Cl. botulinum	TMA	+	+	.
	PSB	+	.	+
Cl. welchii	TMA	+	+	.
	PSB	+	.	+
C. diphtheriae (gravis)	TMA	+	+	.
	Hoyle's	+	.	+
C. xerosis	Loeffler's	+	+	.
H. pertussis	Blood agar	+	.	+
Leuconostoc sp.	**TMA**	+	+	.
M. tuberculosis (laboratory strains of human, bovine and avian, and several freshly isolated from sputa)	Löwenstein-Jensen's	+	.	+
	Dorset egg	+	.	+
M. phlei	TMA	+	+	.
N. gonorrhoeae	Chocolate agar	+	.	+
N. meningitidis	Chocolate agar	+	.	+
P. pestis (a fully virulent and an avirulent strain)	TMA	+	+	.
	PSB	+	.	+
Pf. mallei	TMA	+	(+)	.
Pr. hydrophilus	**TMA**	+	+	.
Ps. pyocyanea	TMA	+	+	.
	Loeffler's	+	+	.
S. typhi	TMA	+	+	.
	MacConkey's	+	.	+
	Desoxycholate	+	.	+
Staph. aureus	**TMA**	+	+	.
	TMA +0·001% gentian violet	−	+	.
Strep. faecalis	TMA	+	+	.
Strep. lactis	**TMA**	+	+	.
Strep. pyogenes (β-haemolytic type)	TMA	+	+	.

Abbreviations. PMM = a medium containing only mannitol, potassium phosphates, a trace of molybdate and well-washed agar. (Topley & Wilson, 1946). TMA = tryptic meat agar. CCY = Gladstone & Fildes, (1940) tryptic casein yeast medium. GL = a chemically-defined medium containing only glucose, citrate and lactate as sources of carbon. (Hills, Belton & Blatchley, 1949). PA = peptone agar. PSB = TMA with addition of glucose and peptic sheep blood.

* For Francis's medium see Stitt, Clough & Clough (1938).

In some cases direct microscopical examination showed that the early growth was not adversely affected by nigrosine. These are indicated above by printing the designation of the medium in **heavy** type.

A plate of tryptic meat agar containing 0·5–1 % of nigrosine is poured and allowed to set. Without further drying it is inoculated at its centre with a small loopful of a suspension of *Strep. lactis* containing *c.* 10^5 organisms/ml. The plate is left with its lid ajar until evaporation and absorption have left no free liquid at the site of inoculation. Then an aluminium ring, being a 5 mm. length cut from a tube 5–7 cm. in diameter, is heated in a flame and dropped concentrically on the surface of the plate. The agar melts locally and seals the ring to the surface. The annular space outside the dam thus formed is partly filled with water, and the plate is placed on three balls of plasticine on the stage of the microscope. A cylindrical brass sleeve (*E*, Fig. 1), is fixed by set screws to the body of the dark ground condenser (*F*), which is applied to the surface of the agar through a loose cover (*G*). The dish and cover are then free to move vertically for focusing; the dish can also slide transversely beneath the cover which remains fixed meanwhile. The plasticine (*C*) allows the dish to be levelled so that the plane of its edge is normal to the axis of the microscope. There is very little loss of vapour from the chamber so formed; in practice the inoculated area of the medium remains steadily in focus for long periods, the slight excess vapour pressure of the water outside the dam over that of the medium itself counteracting any tendency to evaporation from the central area.

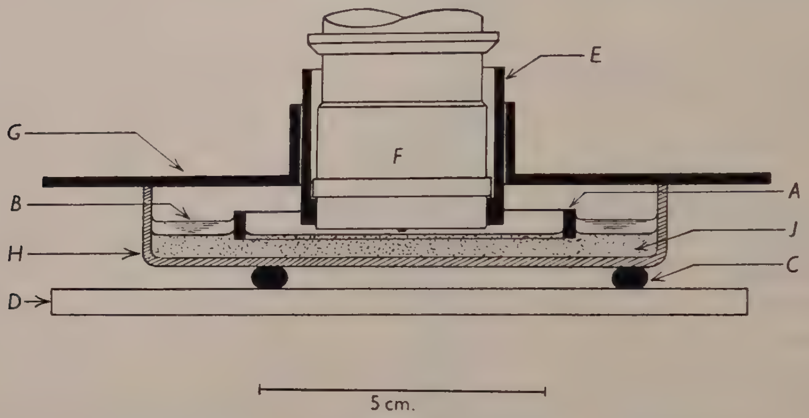

Fig. 1. Section of moist chamber. *A*, aluminium ring; *B*, water; *C*, plasticine balls; *D*, microscope stage; *E*, sleeve fixed to *F*, body of illuminator (enclosing objective); *G*, loose cover sliding on *E* and *H*, Petri dish; *J*, medium.

With oblique incident illumination and the highest available power of the microscope the individual cells of *Strep. lactis* can be seen to grow into chains lying for the most part just beneath the surface of the medium. The organisms are well separated and cell division is easily observed, but no finer details. When the chains contain more than a very few organisms, they begin to buckle as the individuals divide; the mechanical resistance to motion becomes too great to permit perfect longitudinal extension and ultimately each chain forms a very complex pattern. But none ever appears to branch; up to the highest degree of convolution that the eye can follow, a single cell of the inoculum produces a colony consisting of a single chain. Thus the plane of symmetry implied in the mode of cell division of the streptococcus is maintained approximately in its direction through many generations. Pl. 1, fig. 1, shows a typical young colony formed after 3 hr. incubation at 35°.

When conditions are a little drier, growth takes place on the surface of the medium instead of below it, and is best seen by vertical illumination. Pl. 1, fig. 2, shows a young colony growing on a plate dried by storage at 0° for a few days, and inoculated by allowing the organisms to sediment from a cloud produced by spraying, so that no free water was added with the inoculum. Otherwise the conditions were the same as in the previous example. The chain of cells forms the backbone of a ridge whose flanks are composed of the polysaccharide elaborated in quantity by this species.

The post-fission movements of rod-shaped organisms, as described and classified by Graham-Smith (1910) for example, are very easily followed by this method, and the whole architecture of a colony can be traced out. While the method might thus be of some diagnostic value, the variations within any one species may be great, and will depend on the chemical and physical environment and also on purely local conditions determined by the size of the colony already formed.

The growth of *Bacillus subtilis* is interesting in this connexion. When first deposited on a plate, the ellipsoidal spore resembles a diplococcus by oblique illumination, the scattered light apparently radiating from two points near its poles (Pl. 1, fig. 3). When after $1\frac{1}{2}$–2 hr. the vegetative cell begins to grow out, its image is much less bright. The two halves of the spore case do not usually separate completely, though they may do so under pressure from the growing organisms. In any case they retain the high refractivity of the original spore indefinitely (Pl. 1, fig. 4). The subsequent unhampered growth is usually in long chains which form loops and cusps under the longitudinal pressure of growth. At this stage they are scarcely distinguishable from the corresponding forms of *B. anthracis*. As the population density increases, the cohesion of the chains is insufficient to maintain their form; they break apart and the sections slide over each other to produce a colony which consists for the most part of closely packed sheets of organisms. In contrast, *B. anthracis* chains are much stronger and retain their integrity over long distances in spite of considerable mechanical constraint from neighbours (Pl. 1, figs. 5, 6). When the *B. subtilis* colony contains a few hundred organisms, say after 4–5 hr. incubation, motility becomes evident. The close-packed areas are surrounded by a moat of free liquid, a few microns wide and deep, which is presumably extracted from the agar osmotically by the products of metabolism; in it detached organisms swim freely. A few static chains grow out but usually recurve to join the colony again, enclosing loculi which, if small, are filled with a thin layer of liquid. The loculi become populated by motile organisms which arrive from beneath the adjacent sheet of cells; each such organism, after a few minutes violent excursion within the loculus, ultimately rests in contact with the confining wall (either by adhesion or more likely by failure of the motor apparatus) and in this way an extension of the close-packed colony is produced. The whole of this process of piecemeal extension has an aspect of purposeful organization. As the colony grows further it occurs less and less often, and ultimately the edge is without outgrowths, even though the central mass may be in violent swirling motion. Nevertheless, this high degree of organization is largely a product of the environment. With a more dilute agar the organisms can grow to an appreciable depth, and the regularity of packing is lost, whereas if the surface is harder or drier, or both, the organisms lie upon it, and the mechanical resistance to motion principally determines the growth pattern. On a chemically-defined medium containing only citrate, lactate and glucose as sources of carbon (Hills, Belton & Blatchley, 1949), *B. subtilis* of the same strain does not form well-defined chains; the cells are short and soon separate after fission, producing a pattern suggestive of brickwork (Pl. 1, fig. 7). The same pattern is sometimes found on ordinary media, and growth may change from one to the other during the development of a colony.

Use of the Conway Unit for bacterial cultures

The Conway Unit (Conway & Byrne, 1933) is a circular glass dish (diameter *c.* 6·5 cm.; internal depth *c.* 0·8 cm.) with a raised annulus in its floor, originally intended for microchemical analytical work. It is more economical of media, simpler to use, and more generally applicable when used as a culture plate than the Petri dish and aluminium ring. The central cup (diameter *c.* 3·5 cm.; internal depth *c.* 0·6 cm.) is filled almost to overflowing with nutrient medium (5 ml.) so as to present a convex surface; any liquid or solid if required may be placed in the outer annulus. The greater part of the surface of the medium is accessible to the oblique incident illuminator. As an example of the use of the Conway Unit we take the remarkable ability of *B. subtilis* to grow on the surface of a medium quite free from liquid and of great mechanical rigidity.

The centre of a Conway Unit was filled with tryptic meat medium containing 6 % agar. The outer annulus was filled with a mush of solid sodium chloride and water. This mixture maintains a humidity of 75 % over itself. The covered unit was allowed to stand for 3 hr. at 35° and then inoculated with *B. subtilis* spores by exposure to the cloud from a sprayed suspension in a small closed chamber. At this stage a good deal of water had been extracted by the salt from the medium; the sedimented spores evidently stood high on the surface (Pl. 2, fig. 8) which was quite brittle as shown by its cracking under pressure from a glass rod. Although water vapour immediately above such a surface must of course be in equilibrium with it, and therefore not much below saturation, the high rate of evaporation will cause a considerable increase in the concentration of solutes at the surface. The spores were thus in contact with an unusually concentrated medium, but over a small part only of their area. Yet many of them germinated after the normal period and subsequently the reproduction time in the logarithmic phase was about 30 min. as against 20 min. in more ordinary circumstances. The hindrance to motion across the surface was evinced by the production of distorted cells (Pl. 2, fig. 8) and by the throwing up at an early stage of a second layer of cells overlying the first (Pl. 2, fig. 9).

Observations on anaerobes

Anaerobic conditions are not difficult to establish in the closed chamber on the microscope stage. For this purpose we use a circular metal dish to contain the culture vessel (Fig. 2); the objective and condenser are applied through the same loose but close-fitting cover as in Fig. 1. The wall of the dish is pierced with a ring of holes giving upon an annular channel (*B*, *B*, Fig. 2) through which gas can be passed into the interior. The chamber is fed with a slow stream of nitrogen which has been passed through an oxygen absorbent (e.g. titanous chloride). It may also when necessary be saturated with water vapour, or contain an addition of carbon dioxide. The arrangement suffices for the growth of the more exacting anaerobes but the lag phase is likely to be protracted unless steps are taken to remove dissolved oxygen from the medium before inoculation.

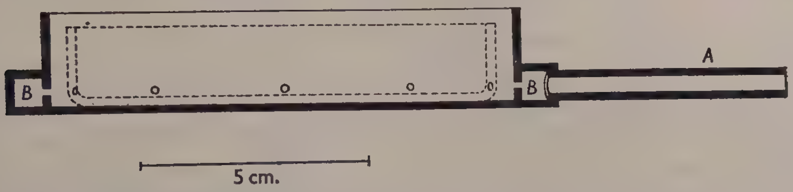

Fig. 2. Section of dish for anaerobic culture under the microscope. *A*, side tube for admission of nitrogen, etc.; *B*, *B*, annular space for distribution of gas.

Pl. 2, fig. 10, shows the edge of a colony of *Clostridium welchii* grown on a nigrosine tryptic meat agar plate in an atmosphere of nitrogen saturated with water. As with *Bacillus anthracis*, the chains rarely break, but the distinguishing feature here is the intertwining of adjacent chains, which do not restrict themselves so rigidly to one plane.

Observation of growth of spores at liquid surfaces

In connexion with the problem of the enhancement of the virulence of *B. anthracis* by detergents (Barnes, 1947), we carried out a few experiments on the growth of spores at liquid surfaces, of which the following illustrates the complementary use of vertical and oblique incident illumination. Spores of *B. subtilis* were dispersed as an aerosol by spraying a clean water suspension. The cloud was allowed to settle for a few minutes on to the surface of tryptic meat broth contained in a well on a microscope slide. When the surface was examined under oblique illumination, the spores were surrounded each by a strong flare; by vertical light they were easily seen to be floating with a considerable portion of their bulk standing out of the surface. They were therefore not much wetted. The experiment was repeated with an aerosol made by spraying a spore suspension containing 5% of 'Tergitol 4', a detergent in which the active compound is principally a branched chain alkyl sodium sulphate (Messrs General Metallurgical & Chemical Corporation, London). Each spore in the cloud thus carried a small quantity of detergent. The sedimented spores in this case were hardly to be seen by vertical illumination, but by oblique illumination gave a well-defined 'diplococcus' image; they had been almost completely wetted on contact with the medium, though the majority remained attached to the surface. This difference in the area of contact was without significant effect on the subsequent development; in both cases vegetative cells appeared beneath the surface after 1½–2 hr. incubation. Unfortunately we were unable to make satisfactory photographs of these phenomena, owing to disturbance of the liquid surface by vibration and convection currents.

Cellophane as a support for growing organisms

Thin permeable membranes have been used from time to time as a support for, or barrier against, growing micro-organisms in order to allow them to be manipulated independently of their culture medium, and vice versa. Thus Gladstone (1948) used Cellophane tubes to separate cultures of *B. anthracis* from their nutrient medium, which was kept flowing over the surface of the Cellophane remote from the organisms, and thus remained sterile; and McKercher & Reed (1948) grew organisms on sheets of Cellophane in order to avoid the use of agar and to enable them to be cleanly stripped from the medium afterwards.

For direct microscopical examination of growing organisms by vertical illumination, Cellophane has some advantages over ordinary media as a support. By comparison with agar it is moderately hard and is not much altered in this respect by changes in the humidity of the surrounding atmosphere. Organisms do not grow into the surface, and can be examined without disturbance under the highest dry powers of the microscope. There is, however, the disadvantage that the normal growth pattern of the colony is obliterated by surface tension forces which cause the organisms to stick together.

Culture on Cellophane under the microscope. We have used Cellophane as a support in two general methods of microscopical observation. One of them—a culture chamber allowing of very complete control of the environment of the organism—will be described in a later communication. The other is intended for the repeated but not continuous examination of young growths, more especially when many specimens are being studied at the same time. It consists of a standard 'Bijou' (¼ oz.) bottle (Messrs Gallenkamp, London) with a Cellophane window in the screw cap. The bottle contains the growth medium and the outer surface of the window is inoculated with the organism under test. A disk of thin rubber is placed inside the screw cap on the thick washer supplied and a 1 cm. hole is punched simultaneously through both layers of rubber and the cap itself, using a sharp-edged, deeply recessed punch against a sheet of fibre. The washers are removed, and the thinner one replaced first in the cap. This is followed by a disk of Cellophane, 20–25μ. thick, and then the thicker washer (Fig. 3). One or two ml. of medium are put in the bottle and the cap screwed on lightly; the whole is then autoclaved. The outer surface of the Cellophane may then be inoculated. The inoculum is allowed to evaporate nearly to dryness, the bottle inverted in a boiling tube or other narrow vessel, and incubated in this position. At first the Cellophane is wrinkled, but as the medium warms up and its vapour pressure increases, the window forms a taut dome on which it is easy to focus the microscope. When the bottle is removed for examination, the Cellophane is usually seen to be covered with small droplets of liquid; these evaporate rapidly when the bottle is in the upright position, and the organisms on the surface are very clearly seen under the microscope by vertical illumination. There is sufficient liquid underneath the Cellophane to prevent complete drying during a short examination, after which the bottle may be restored to the inverted position and growth allowed to proceed as before. Even if only a single observation has to be made, e.g. for presence or absence of growth, there is still a considerable advantage in the short time normally elapsing between inoculation and the formation of recognizable colonies, and in the rapidity with which a long series of cultures may be examined. In experiments lasting only a few hours, the usual sterile precautions are for most purposes superfluous; the chance of seeing a contaminant under the microscope is remote, and infection of the medium can in its early stages rarely affect the behaviour of the organisms being studied.

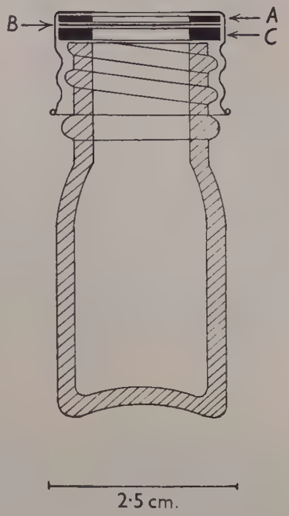

Fig. 3. Section of screw-cap bottle with Cellophane window. *A*, thin rubber washer; *B*, Cellophane disk; *C*, thick rubber washer.

In this way we have been able to study the action of certain antibiotics. By comparison of serial dilutions of standard and unknown preparations it is possible to make estimates of concentration within a few hours. The accuracy is rather greater than in an all-or-nothing growth test, since the degrees of aberration produced in the test organism near the critical concentration can be assessed roughly. Pl. 2, fig. 11, shows some forms of *B. subtilis* grown for 3 hr. on Cellophane over peptone water containing 0·003 units penicillin/ml. In one case the two halves of the spore residue have divided, and have remained attached one to each end of a short chain of cells. More remarkable is the presence of two very distorted growths, one undergoing lysis, in these circumstances providing evidence that the cells are quite flaccid. Under the actual conditions of growth, the organisms are immersed in droplets of liquid. When the bottles are returned to the upright position these droplets evaporate and surface tension tends to keep the organisms within their boundaries. The consequent bending of the organisms can be seen to occur at this stage. Only a moderate curvature, if any, is produced in healthy growths (compare Pl. 2, fig. 12).

Among the limitations on the use of Cellophane is its small pore size; Madras, Mason & McIntosh (1949) estimated the average pore diameter to be about 2·8 mμ. only. This is a severe restriction on the size of molecules which will pass through; penicillin passes easily, lysozyme very slowly, haematin not at all. But no other membranes that we have been able to test will replace Cellophane. Nylon and various cellulose esters are less readily wetted by water, and films of these substances will not permit the passage of medium unless there is liquid on both sides. Membranes from animal tissues and eggs are otherwise suitable, but have too coarse a structure to present a smooth background.

The impermeability of Cellophane to large molecules is demonstrated in our last example. Mechanically defibrinated horse, cow or human blood will not support growth of *B. subtilis*. Spores deposited from the air on to the surface swell and break open, but then appear to become encysted, and grow no further. Up to at least 5 hr. after inoculation they remain viable, and when transferred to an ordinary medium will grow normally. Blood to which citrate or oxalate has been added will support growth, though rather poorly. If, however, the spores are separated by Cellophane from mechanically defibrinated blood, they germinate normally and subsequently grow very freely (Pl. 2, fig. 13). It will be noted that short detached vegetative forms are produced (cf. Pl. 1, fig. 7). The inhibitor is thus of fairly large size; it may be produced in the clotting process or removed by anticoagulants.

We are indebted to Messrs British Cellophane Ltd., Bridgwater, Somerset, for a large sample of good quality Cellophane; to our instrument maker, Mr G. Clement; and to Miss Nora Harris, who took the photographs. Publication is by permission of the Chief Scientist, Ministry of Supply.

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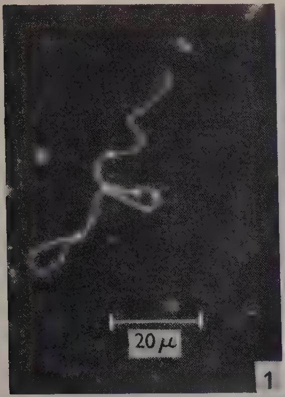

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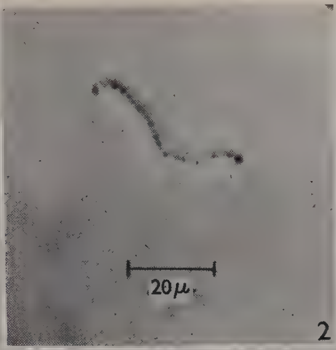

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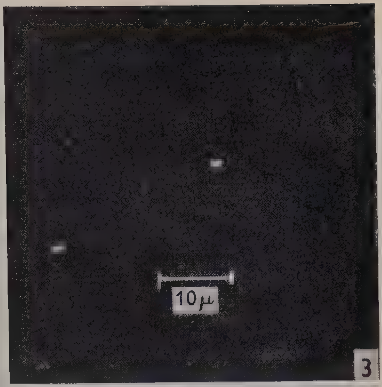

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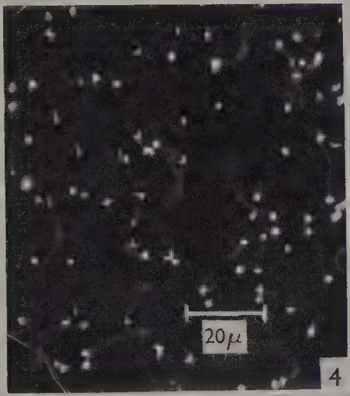

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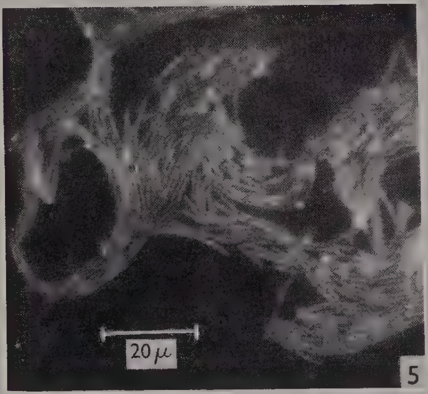

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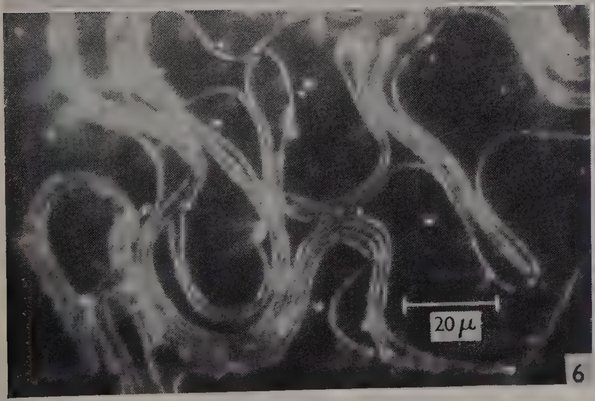

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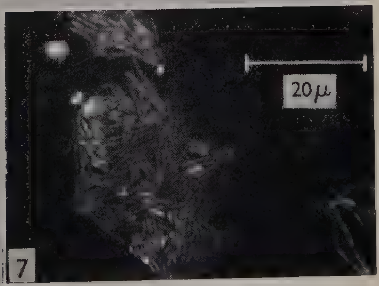

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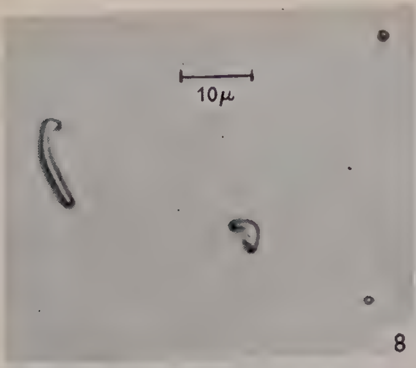
Figs. 1–7

T. W. PEARCE AND E. O. POWELL—NEW TECHNIQUES FOR STUDYING GROWING BACTERIA. PLATE 1



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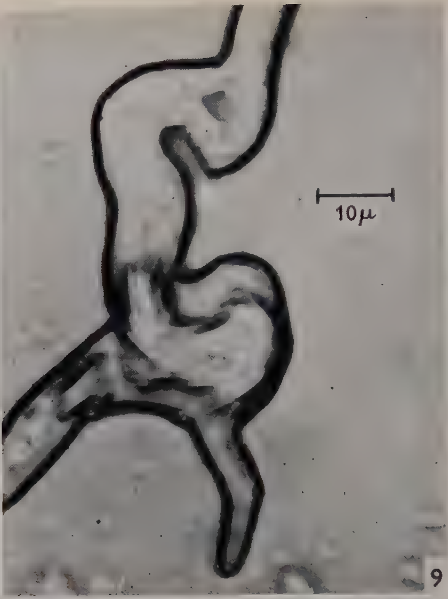

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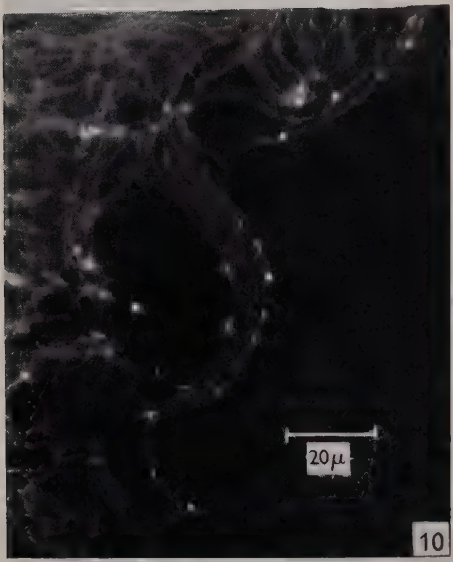

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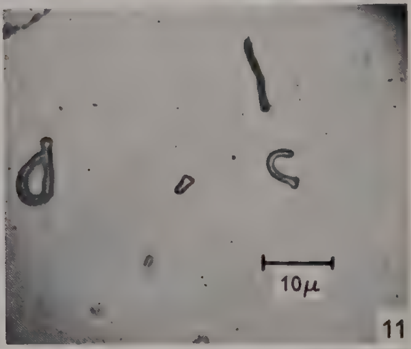

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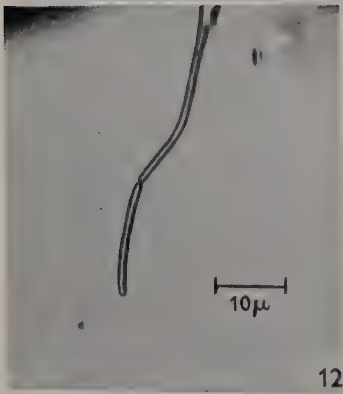

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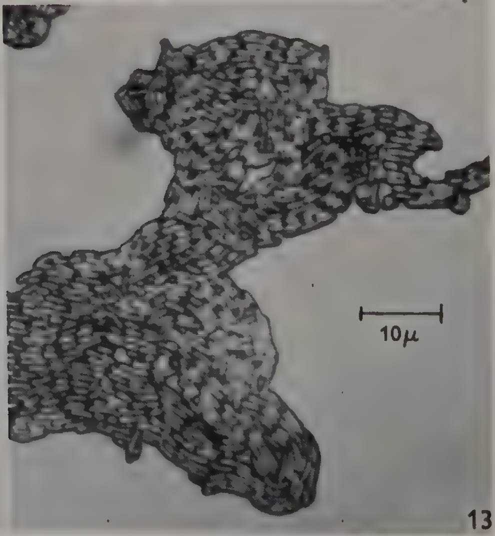

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Figs. 8–13

T. W. PEARCE AND E. O. POWELL—NEW TECHNIQUES FOR STUDYING GROWING BACTERIA. PLATE 2

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Strep. lactis.* 3 hr. at 35° on nigrosine tryptic meat agar plate. Oblique incident illumination. Objective: Cooke, ×45, 0·65 N.A. achromat.

Fig. 2. *Strep. lactis.* As fig. 1 but on drier plate and with vertical illumination.

Fig. 3. *B. subtilis.* Spores on nigrosine medium, showing apparent diplococcal form. Optics as fig. 1.

Fig. 4. *B. subtilis.* Spores and young vegetative forms after 2 hr. at 35° on nigrosine medium. Optics as fig. 1.

Fig. 5. *B. subtilis.* Part of colony 6·5 hr. old on nigrosine peptone agar. Optics as fig. 1.

Fig. 6. *B. anthracis.* Part of colony 6·5 hr. old on nigrosine peptone agar. Optics as fig. 1.

Fig. 7. *B. subtilis.* Colony 3 hr. old, grown on a chemically-defined medium. Short detached forms embedded in medium. Optics as fig. 1.

PLATE 2

Fig. 8. *B. subtilis.* Spores and young growths on a very dry medium, showing a distorted cell and residues of spore-case. Vertical illumination. Objective: Beck, ×40, 0·95 N.A. apochromat.

Fig. 9. *B. subtilis.* Young colony on very dry agar medium, illustrating the piling up of cells at an early stage. Maximum contrast has been used in reproducing the photograph so as to bring out details within the boundary of the colony. Vertical illumination. Objective: Cooke, ×45, 0·65 N.A. achromat.

Fig. 10. *Cl. welchii.* 16 hr. at 35° on nigrosine tryptic meat agar plate. Edge of colony, characterized by twisting together of adjacent chains. Optics as fig. 1.

Fig. 11. *B. subtilis.* 3 hr. growth on Cellophane over peptone water containing 0·003 units penicillin/ml. Abnormal forms. Optics as fig. 9.

Fig. 12. *B. subtilis.* Outgrowth from a young colony grown on Cellophane over peptone water. Illustrating rigidity of healthy organism. Optics as fig. 8.

Fig. 13. *B. subtilis.* Colony of short detached forms grown on Cellophane over mechanically defibrinated human blood. Optics as fig. 9.

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The Application of Electron Microscopy to the Study of Plant Viruses in Unpurified Plant Extracts

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SUMMARY: Rods of variable lengths occurred in sap from plants infected with tobacco mosaic, cucumber mosaic, potato *X*, potato *Y*, henbane mosaic, tobacco etch, and cabbage blackring viruses; the first two were about 15 mμ. wide and appeared rigid, the others were about 10 mμ. wide and apparently flexible. Sap from plants infected with tomato bushy stunt, tobacco ringspot and two tobacco necrosis viruses contained spherical particles about 26 mμ. in diameter; two particles, one about 18 mμ. and the other about 37 mμ. in diameter occurred in sap from plants infected with a third tobacco necrosis virus. No specific particles were identified in sap from plants infected with tomato spotted wilt, potato leaf roll, cauliflower mosaic, tomato aspermy, sugar beet mosaic and sugar beet yellows viruses. Serologically related strains of any one virus were morphologically indistinguishable, but this has little diagnostic value because so also were some unrelated viruses.

Electron microscopy has so far been mainly restricted to the study of purified plant viruses, for which there are alternative methods of assessing particle size. Since the development of the shadow-casting technique (Müller, 1942; Williams & Wyckoff, 1944), there has been no difficulty in detecting particles of such viruses as tobacco mosaic, potato *X* and tomato bushy stunt in clarified infective sap, but there has been no systematic attempt to apply the technique to less stable viruses about whose sizes and shapes little is known. The work described in this paper was begun to see whether electron microscopy was generally applicable to unpurified preparations of plant viruses and to assess its value for identification purposes.

MATERIALS AND METHODS

The following virus-host combinations have been studied; tobacco mosaic, potato virus *X*, potato virus *Y*, tomato spotted wilt, tomato aspermy (Blencowe & Caldwell, 1949), henbane mosaic, cucumber mosaic, tobacco etch and the tobacco necrosis viruses in tobacco (*Nicotiana tabacum*), var. White Burley; tomato bushy stunt, tomato spotted wilt and potato paracrinkle in tomato (*Lycopersicum esculentum*), var. Kondine Red; cauliflower mosaic and cabbage blackring in turnip (*Brassica napus*); cabbage blackring in *Nicotiana glutinosa*; sugar beet mosaic and sugar beet yellows in sugar beet (*Beta vulgaris*); potato leaf roll and potato paracrinkle in potato (*Solanum tuberosum*), several varieties; potato leaf roll in *Datura stramonium*.

Various methods of making extracts from infected leaves were tried, of which the following gave the most generally satisfactory results. Leaves were picked when showing severe symptoms, and the bulk of the mid-ribs were cut out and discarded. The laminae were lightly dusted with Na_2HPO_4, ground with a pestle in a mortar and the sap expressed through muslin. After lying for some hours at −10°, the sap was thawed, centrifuged for 10 min. at 7000 r.p.m. and the supernatant fluid used immediately for making specimens for electron microscopy. Samples of the fluid were also tested by inoculation to a suitable range of differential host plants to ensure their freedom from contaminating viruses. None was ever found, and we have no reason to think that our preparations contained any virus except the one named. Samples of clarified sap from uninfected plants of the same species were also made by the same method.

The mounts for electron microscopy were made by placing small drops of the clarified sap on collodion-covered specimen grids and removing the fluid after a minute with a micro-pipette. To remove salts, the mounts were dipped in distilled water, and dried on hard filter-paper. They were then shadowed with about 8 A. thickness of palladium, which was cast at an angle of about 12 degrees from the plane of the mount. The electron microscope was an R.C.A. model B, fitted with a three-electrode gun, and was used with an accelerating potential of 50 kV.

RESULTS

The suitability of different species of plant for this kind of work varied widely, for whereas sap from some when mounted for electron microscopy contained little background material that interfered with the identification of possible virus particles, the sap from others contained much. Sap from solanaceous plants except the potato, after treating with phosphate and freezing, was more suitable than that from other species. Potato is particularly unsuitable, because sap from uninfected plants of all the varieties we have tried contains rod-like particles resembling those of some viruses (Bawden, Kassanis & Nixon, 1950).

Virus-host combinations with which no specific particles were identified

Our results can conveniently be divided into two groups; the preparations of sap from infected plants in which we found no particles distinct from those that also occurred in similar preparations from healthy plants, and those in which we found particles specific to the infected plants. The first group contains tomato spotted wilt, potato leaf roll, sugar beet mosaic, sugar beet yellows, cauliflower mosaic and tomato aspermy viruses. There is therefore still no information bearing on the size and shape of these virus particles. Failure to detect any particles that might be the viruses themselves may have different explanations with different virus-host combinations. With some, the virus content of sap is probably too small for the technique to be applicable. The limiting concentration for success depends on the shape of the particles and on the composition of the medium from which they are dried in making mounts for examination in the electron microscope. The rod-shaped particles of tobacco mosaic virus can be found without great difficulty when purified preparations are diluted in water to concentrations of 0·01 mg./l., and the spherical particles of tomato bushy stunt virus up to 0·01 mg./l. When diluted in clarified sap from healthy tobacco plants, however, and the mounts washed to free them from salts in the manner described, the limiting concentration at which tobacco mosaic virus particles are readily detected is about 1 mg./l. and tomato bushy stunt 10 mg./l. The effects of the mounting medium in increasing the limiting concentration are partly attributable to the removal of some virus particles when the mounts are washed with water, and partly to the fact that other materials in sap make it difficult to identify individual particles when they are widely separated. Individual particles of tobacco mosaic virus are more easily detected than those of tomato bushy stunt virus because elongated rods are more distinctive than spheres of comparable widths. This is especially so with clarified sap in which deposits of normal plant materials are more likely to obscure spheres than rods; also sap from uninfected plants of all species contain spherical particles resolvable by the electron microscope. From frozen sap of *Nicotiana* sp., tomato and turnip, these are smaller than particles of any of the viruses we have studied. Clarified sap from uninfected potato plants contains rod-like particles of unknown composition (Bawden *et al.* 1950), but we have seen none in the clarified frozen sap of any of the other species of uninfected plants we have examined.

A virus content of less than about 10 mg./l. if the particles are spherical and 1 mg./l. if they are rod-shaped, may explain our failure to detect specific particles in sap from plants infected with tomato spotted wilt, potato leaf roll, sugar-beet mosaic and cauliflower mosaic viruses, but this explanation is inadequate with sugar beet yellows. Clarified sap from infected sugar-beet leaves contains a specific antigen that precipitates with antiserum up to dilutions of 1/64 (Kleczkowski & Watson, 1944). The precipitate is of somatic type, resembling that given by viruses with spherical particles, and the precipitation end-point suggests a virus content in sap of more than 100 mg./l., similar to that reached by tomato bushy stunt virus. The antigen sediments in centrifuge fields of 30,000 *g* and so should be amply large to be resolved in the electron microscope. Our failure to identify any particles that might be sugar-beet yellows virus probably means that the virus has spherical particles of a similar size to those that occur in normal sugar-beet leaves. Unlike electron micrographs of clarified sap from the solanaceous plants we have examined, those of sap from uninfected sugar beet give a field covered thickly with spherical particles about 10 mμ. in diameter. Like the specific antigen from infected leaves, these particles sediment in the ultracentrifuge, and only by serological tests have differences yet been detected between sap from infected and healthy sugar beet plants. The quantities of large particles of normal components also may explain our failure to detect sugar beet mosaic virus; we have occasionally had suggestions that sap from infected plants contained rod-like particles, but these were too heavily overlain with other particles for any certain identification.

Virus-host combinations containing specific particles

In sap from plants infected with the other viruses, we have found particles that are absent from similar preparations made from uninfected plants. These are assumed to be the virus particles, and we shall refer to them as such, but in the absence of any proof of their infectivity, it must be stressed that this is an assumption and that their identity remains unestablished. Several strains of potato virus X have been found to have rod-like tenuous particles about 10 mμ. wide and of greatly variable lengths. The manner in which these aggregate to form extensive rod- and net-like structures has been described by Kleczkowski & Nixon (1950). Three strains of potato virus Y, namely, our stock virulent culture, (Pl. 1, fig. 1) potato virus C (Pl. 1, fig. 2) (Bawden & Kassanis, 1947) and tobacco veinal necrosis virus (Pl. 1, fig. 3) (Smith & Dennis, 1940), which are serologically related but cause widely different diseases in different hosts, were also rod-like particles about 10 mμ. wide and variable lengths. They also appeared to resemble potato virus X in being tenuous, capable of linear aggregation and becoming intertwined, (Pl. 1, fig. 2) but the concentration of these particles was much smaller than with potato virus X, and the phenomenon has not been studied in any detail. The results are in keeping with those from attempts to purify this virus, which showed that concentrated preparations gave the phenomenon of anisotropy of flow and that the virus occurred at about one-fiftieth of the concentration of potato virus X (Bawden & Pirie, 1939). Particles similar to those of potato virus Y, and occurring in similar quantities, were also found in sap from plants infected with potato paracrinkle, two strains of henbane mosaic, tobacco etch, and cabbage blackring viruses. It is probable that our cabbage blackring virus is similar to the *Brassica nigra* virus described by Takahashi (1949). In sap from plants infected separately with two different strains of cucumber mosaic virus, rod-like particles were also found, but these differed slightly from those already described. They were again of variable lengths, but resembled those of tobacco mosaic virus in being about 15 mμ. wide, and in having an appearance of rigidity that contrasts with the seeming flexibility of those with narrower particles. They occurred in numbers comparable to those found in sap from tobacco mosaic plants diluted about 1/1000, and there is also a factor of this order between the dilution end points at which sap from tobacco plants infected with the two viruses ceases to cause infections in tobacco.

The other viruses we have studied all appear to have spherical particles, which, except for tobacco ringspot virus, is in keeping with what is known of their physical properties and serological behaviour. Tobacco ringspot, from studies of physical properties of purified preparations, has been variously stated to have rod-shaped particles 183 mμ. long and 10·4 mμ. wide (Stanley, 1938) and a spherical particle of diameter 19 mμ. (Stanley, 1939); our results suggest a spherical particle with diameter about 26 mμ. (Pl. 2, fig. 9). This is similar to the size of tomato bushy stunt virus (Pl. 2, fig. 14) and also to the tobacco necrosis viruses which Bawden & Pirie (1942) called tobacco II, tobacco VI and potato (Pl. 2, figs. 11–13). These three all have the same sedimentation constant. The two last are related strains but are serologically unrelated to the first. No cultures of these viruses are now maintained, and our pictures were made from clarified sap that had been kept in the refrigerator for more than 5 years. The sap was not infective but was still serologically active. The particles are less well defined than those of the spherical viruses examined in fresh sap, and it may be that they had been denatured by ageing. Sap from tobacco leaves infected with the Rothamsted tobacco necrosis virus differed from any other in that it contained specific spherical particles of two sizes; one is about 18 mμ. and the other about 37 mμ. in diameter. In the plate (Pl. 2, fig. 10) we show a micrograph of a partially purified preparation of this virus because our pictures of these particles in clarified sap are unsuitable for reproduction. These two sizes presumably correspond to those shown by Ogston (1942) to have sedimentation constants of 240 and 51 S. Particles of similar sizes occur in sap from plants infected with bean stipple-streak virus, which is serologically related to the Rothamsted tobacco necrosis virus (Bawden & van der Want, 1949). The possible relationships between these two particles have been discussed by Bawden & Pirie (1950).

MORPHOLOGY AND CLASSIFICATION

Our results show that by applying the technique of shadow casting to mounts of clarified infective sap, electron microscopy can be used to determine the approximate sizes and shapes of the particles of some plant viruses that are insufficiently stable and occur at too high dilutions to be readily purified. It is unlikely to be applicable to all viruses, but by modifications of our technique, for example, by better methods of washing the mounts or by using methods of clarifying infective sap that remove more of the normal plant components but not the virus particles, it may be extended to many more and probably to some with which we have so far failed. The fact that clinically distinct viruses, which are now grouped as strains because they are serologically related, and mutually antagonistic when present together in infected plants, have particles of the same size and shape suggests, at first sight, that electron microscopy may be valuable in classifying viruses. However, many serologically unrelated viruses with widely differing general properties have appeared to be indistinguishable morphologically, and, as with ordinary microscopy and bacteria, the value of electron microscopy seems likely to be restricted to dividing plant viruses into a number of broad groups. Consistent differences in particle size and shape between viruses will be useful and will help to distinguish between clinically similar types, but the fact that two viruses have particles of the same size and shape is no evidence of relationship.

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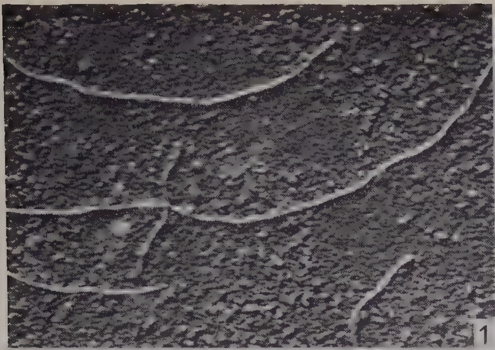
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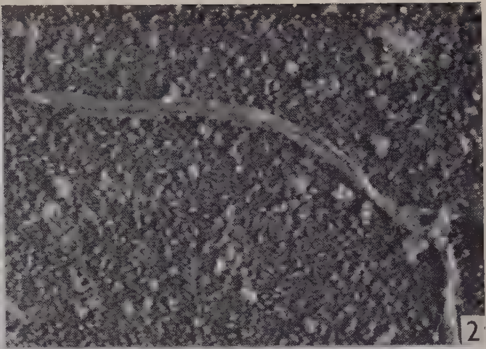
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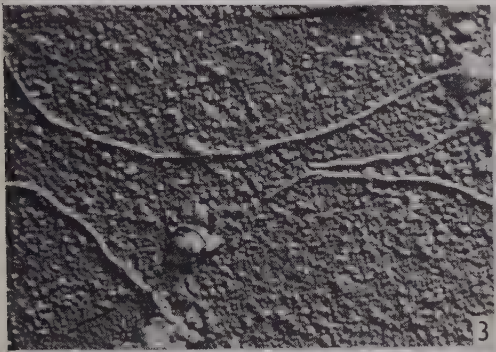
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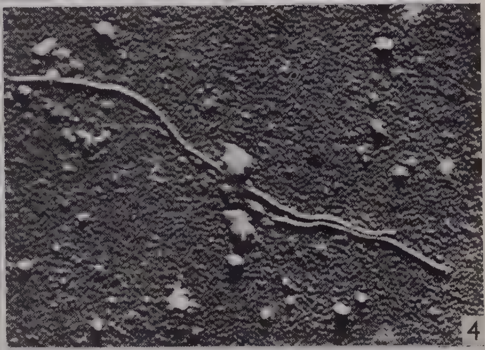
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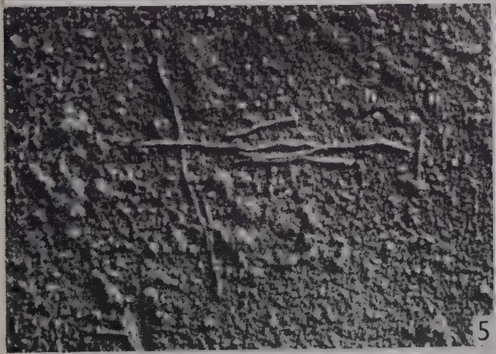
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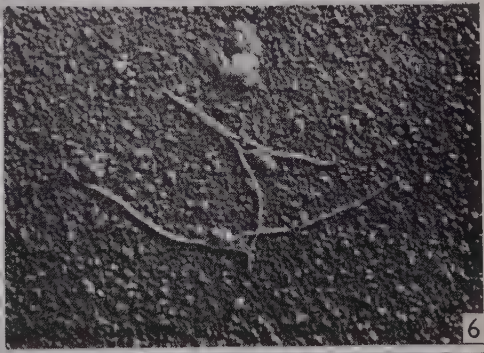
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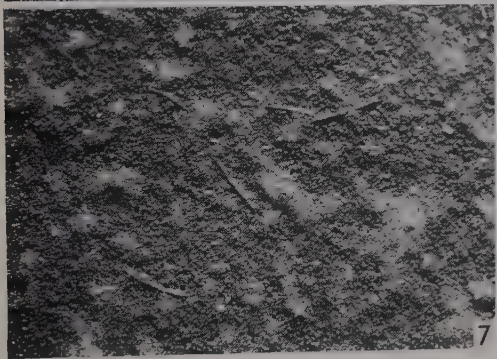
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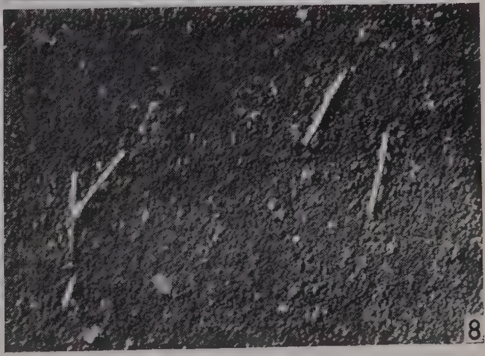
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Figs. 1–8

F. C. Bawden and H. L. Nixon—Electron microscopy of unpurified viruses. Plate 1

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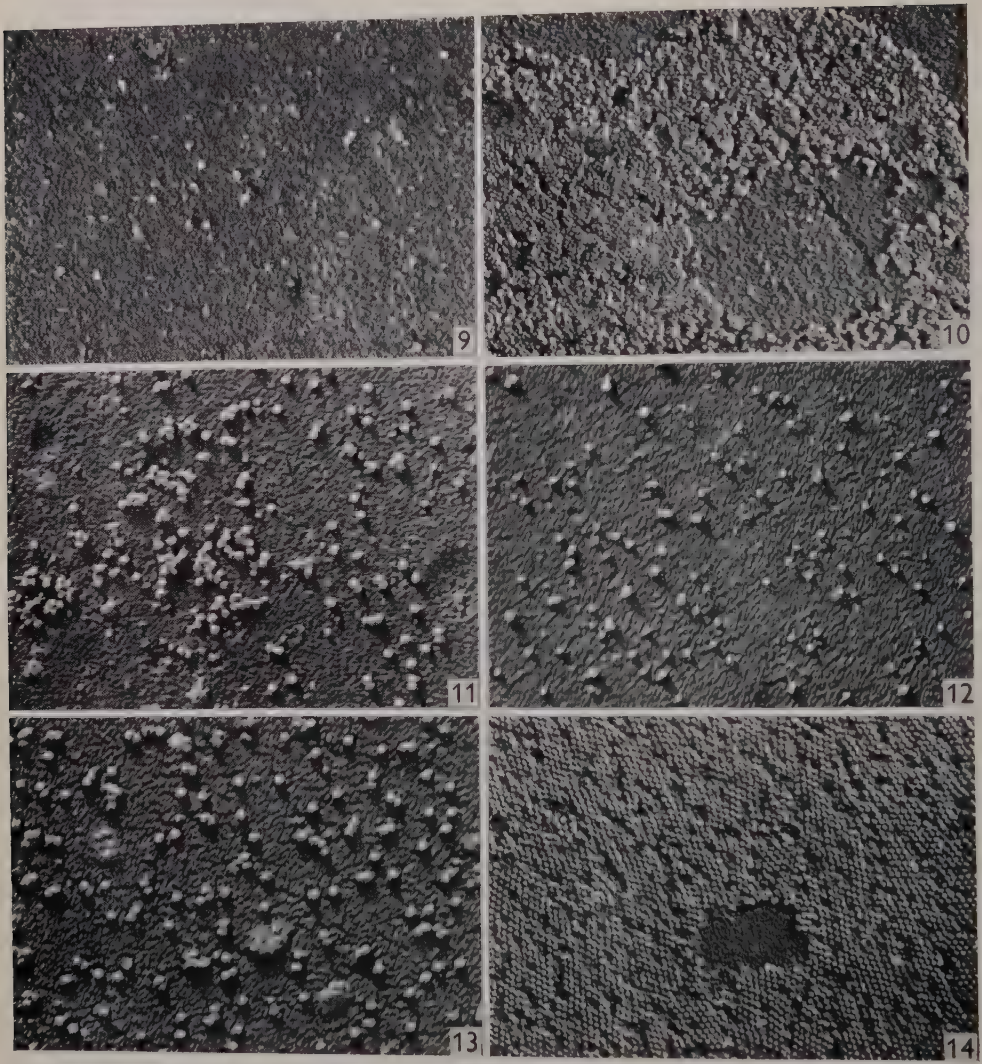

Figs. 9–14

F. C. Bawden and H. L. Nixon—Electron microscopy of unpurified viruses. Plate 2

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EXPLANATION OF PLATES

All mounts were made from clarified sap unless otherwise stated, and all were shadowed with about 8 A. of palladium. The final magnification has been adjusted in the photographic work to × 30,000.

Plate 1

Fig. 1. Potato virus *Y* in tobacco.

Fig. 2. Potato virus *C* (serologically related to potato virus *Y*) in tobacco.

Fig. 3. Tobacco veinal necrosis virus (serologically related to potato virus *Y*) in tobacco.

Fig. 4. Severe etch virus in tobacco.

Fig. 5. Cabbage blackring virus in turnip.

Fig. 6. Henbane mosaic virus in tobacco.

Fig. 7. Cucumber mosaic virus (strain causing a bright yellow mottle) in tobacco.

Fig. 8. Cucumber mosaic virus (strain causing severe necrosis) in tobacco.

Plate 2

Fig. 9. Tobacco ringspot virus in tobacco.

Fig. 10. Tobacco necrosis virus (Rothamsted culture) mount from virus preparation which had been partially purified with the ultracentrifuge.

Fig. 11. Tobacco necrosis virus, tobacco II culture of Bawden & Pirie (1942).

Fig. 12. Tobacco necrosis virus, potato culture of Bawden & Pirie (1942).

Fig. 13. Tobacco necrosis virus, tobacco VI culture of Bawden & Pirie (1942).

Fig. 14. Tomato bushy stunt virus, mount from partially purified preparation.

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Bacteriolysis by a Species of *Streptomyces*

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SUMMARY: The lysis of dead *Bacterium coli* by concentrates prepared from filtrates of a *Streptomyces* sp. is probably due to proteolysis. In any event the lysis can be accounted for by proteolysis, even if the action of other bacteriolytic enzymes cannot be excluded. The streptomyces preparation contains at least two proteolytic enzymes, one digesting casein and the other digesting protein(s) present in *Bact. coli*. The substrates of the autolytic enzymes of *Bact. coli* are probably different from those of the streptomyces enzymes.

The bacteriolytic activity of actinomycetes has been discussed by several authors (e.g. Waksman, 1947; Welsch, 1947; Jones, Swallow & Webb, 1948). Dead Gram-negative, dead Gram-positive, living Gram-positive, and living Gram-negative organisms, in that order of susceptibility, are said to be lysed by sterile culture-filtrates of certain species of actinomycetes. The lysis of living organisms is open to doubt because it is difficult, if not impossible, to determine whether the organisms that are lysed in a suspension containing living organisms are living or not. The lysis of organisms by cultures of actinomycetes is thought to consist of two stages: the killing of the organisms by an antibiotic, and the lysis of the dead organisms by a lytic agent.

It seemed to us that this lytic agent might be one or more proteolytic enzymes. Welsch (1947) contended that the bacteriolytic activity of *Streptomyces albus* G was not due to proteolysis only. He stated that 'des actinomycétines concentrées, mais encore très impures, ont le même activité bactériolytique par mgr. d'N protéique que des solutions de trypsine cristallisée, sans posséder cependant leur activité protéolytique'. The proteolytic activities of the streptomyces preparation and the crystalline trypsin were compared in their action on gelatin, casein and coagulated serum. This does not exclude the possibility that the streptomyces preparation might have contained proteolytic enzymes, relatively inactive towards these particular proteins, but active against other proteins that might be present in the lysable micro-organisms.

Jones *et al.* (1948) showed that culture filtrates of various species of actinomycetes used by them contained at least two proteolytic enzymes. They stated that the bacteriolysis by actinomycetes was due to these enzymes, but did not show that proteolysis took place during bacteriolysis.

We have shown that the bacteriolytic activity of a *Streptomyces* sp. isolated from the soil can be accounted for by its proteolytic activity, and that its proteolytic activity is due to at least two enzymes, one of which is caseinolytic and the other capable of digesting the protein(s) present in certain bacteria.

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METHODS

Isolation of culture. Soil samples were selected at random from fields in Oxford. They were mixed with distilled water and allowed to settle at room temperature. Water-agar plates incorporating living *Staphylococcus aureus* and dead *Bact. coli* were inoculated with the supernatant fluid and incubated for 3 days at 37°. Colonies of presumptive actinomycetes that had clear zones of lysed *Staph. aureus* or *Bact. coli* around them were isolated and transferred for sporulation to mineral agar slopes containing 2 % sucrose. Altogether ten strains were isolated; four were highly active against living and dead *Staph. aureus* and *Bact. coli*; two were moderately active; four were inactive. The most active strain was chosen for further study and was identified by Dr Kathleen Crawford as a species of *Streptomyces*.

Production of lytic filtrates. After several trials a tryptic digest of casein was found to be suitable for the production of bacteriolytically-active culture filtrates on a large scale. Casein (300 g.; British Drug Houses Ltd., light white) is mixed with 300 ml. ethanol and uniformly suspended by stirring in 3 l. of warm tap-water. The pH is adjusted to 8·5, 8 g. commercial crude trypsin (Harrington) are added, and the mixture is maintained at 50–55° for 12–18 hr. During the first few hours the pH is maintained at 8·5 by frequent additions of 10 N-NaOH. After 6–9 hr. a further 8 g. of crude trypsin are added. Chloroform (1 %) is added to prevent the growth of bacteria. The digestion is complete, after 12–18 hr., when a sample no longer precipitates with an equal volume of 10 % (w/v) trichloroacetic acid. The digest is then boiled for 1 hr., filtered, and made up to 3 l. The final medium had the following composition: $NaNO_3$, 2·0 g.; K_2HPO_4, 1·0 g.; KCl, 1·0 g.; $MgSO_4.7H_2O$, 1·0 g.; tryptic digest of casein (10 %), 40·0 ml.; water to 1000 ml.; the pH is 7·8–8·0. A final concentration of 0·4 % (v/v) of trypsin-digested casein is the optimum for the production of bacteriolytically active filtrates. With higher or lower concentrations of casein digest less active filtrates are obtained.

The medium was distributed in 4 or 8 l. quantities in 5 or 10 l. Pyrex bottles, inoculated with a 4-day growth of the *Streptomyces* sp. on the same medium, and incubated at 24° or 37°. During incubation the cultures were aerated with one volume of air per volume of culture fluid per minute. About the same concentration of lytic substances is reached whether the cultures are incubated for 5–6 days at 24°, or for 48 hr. at 37°.

Concentration of lytic agent. The cultures were clarified in a Sharples centrifuge and the clear supernatants concentrated about 30-fold by evaporating-off the water (12–14 l./hr.) under reduced pressure at 36° (van Heyningen, 1949). The lytic substances were precipitated from the concentrate by the addition of $(NH_4)_2SO_4$ to 0·65 saturation. The precipitate was suspended in a small volume of water, dialysed against running tap-water for 2 days, and freeze-dried, the dried product being kept in a desiccator. Stock concentrated solutions of the crude product in 66 % glycerol were kept in a refrigerator. These solutions retain their activity without loss for several months.

Estimation of proteolytic activity. Two substrates were used: (i) a 1 % (w/v) solution of casein (British Drug Houses Ltd., light white) in 0·67 M phosphate buffer (pH 8·0); (ii) a 1 % (dry wt./v) suspension of *Bact. coli* in the same buffer. The casein solution was made by suspending 1 g. casein in 100 ml. buffer and heating in a boiling water-bath for 10 min. The resulting solution was turbid. The bacterial suspension was made as follows: over-night cultures of *Bact. coli* on nutrient agar in Roux bottles were harvested, washed three times with distilled water and resuspended in distilled water. The dry weight of 1 ml. samples of the suspension was determined, and the organisms spun down and resuspended in buffer to make a 1 % (dry wt./v) suspension. This suspension was then autoclaved at 120° for 20 min.

The method used for estimating proteolytic activity was a modification of Kunitz's (1947) method. Equal volumes (10 ml.) of enzyme solution in phosphate buffer (pH 8·0) and substrate solution or suspension, both previously warmed to 37°, are mixed together. Duplicate 4 ml. samples are immediately withdrawn (0 min.) and each is discharged into 4 ml. of 6 % (w/v) trichloroacetic acid solution. The rest of the mixture is incubated at 37° for 20 min., when two more 4 ml. samples are withdrawn and discharged into trichloroacetic acid. The trichloroacetic acid-precipitated samples are then filtered through Whatman No. 1 paper and the optical density of the filtrates at 280 mμ. (D_{280}) is determined in a Beckman DU photoelectric absorption spectrophotometer. The value for the increase in D_{280} is then translated into units of proteolytic activity by means of a *standard curve*. A unit of proteolytic activity (Cas. units where casein is the substrate; Col. units where *Bact. coli* is the substrate) is defined as that amount of enzyme which, under the conditions described, will hydrolyse the substrate at such a rate that in 20 min. there is an increase of 0·30 in the D_{280} of the trichloroacetic acid filtrate. The *standard curve* is derived from the *activity curve*, which is constructed as follows: increasing concentrations of enzyme are incubated with the substrate for 20 min. and the values for increase in D_{280} of the resulting trichloroacetic filtrates are determined in the usual way. These are then plotted against the relative enzyme concentrations, as is shown in Fig. 1 for the activity curves for the crude streptomyces preparation (A), crude commercial trypsin (B), and crystalline trypsin (C), acting on *Bact. coli* suspension. Fig. 2 shows the standard curves derived from these curves. A tangent is drawn to the initial portion of each activity curve; from this tangent the amount of proteolytic agent causing an increase of 0·30 in the D_{280} of the trichloroacetic acid filtrate is read. This is the unit. The data on a smooth line passing through the points of the activity curve are then replotted on the standard curve so that multiples of units fall on convenient increments of the abscissa.

Objection might be raised on the grounds that the liberation from autoclaved *Bact. coli* of trichloroacetic acid-soluble substances absorbing at 280 mμ. might not be a measure of the action of a proteolytic enzyme, but of an enzyme liberating substances like purines and pyrimidines that absorb ultra-violet light. However, such an objection does not apply to this work. The absorption spectra of the trichloroacetic acid-soluble material resulting from the action of (*a*) crystalline trypsin on autoclaved *Bact. coli*, (*b*) the streptomyces preparation on autoclaved *Bact. coli*, (*c*) crystalline trypsin on casein, and (*d*) the streptomyces preparation on casein were all very similar, as can be seen from Fig. 3. These spectra were determined by Dr G. V. R. Born. None of the preparations had absorption maxima in the ranges not shown in the figure. The crude streptomyces preparation liberated trichloroacetic acid-soluble substances from autoclaved *Bact. coli* with much the same absorption spectrum as those liberated by crystalline trypsin from casein. These curves show that it would probably be better to measure absorption at 275 mμ. rather than at 280 mμ., as is usual in the estimation of proteolytic activity.

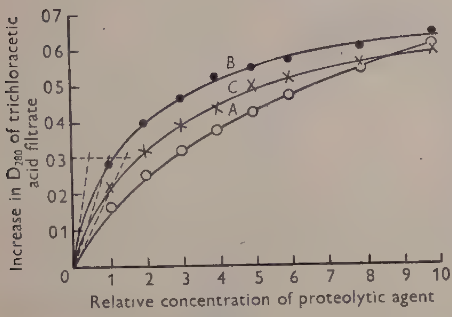

Fig. 1

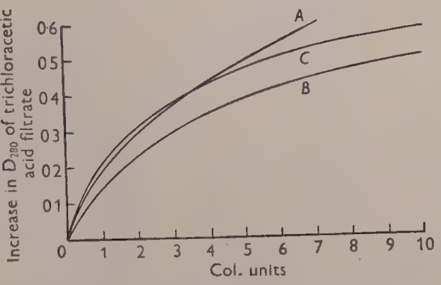

Fig. 2

Fig. 1. Proteolytic activity curves for the streptomyces preparation (*A*), crude trypsin (*B*), and crystalline trypsin (*C*), with autoclaved *Bact. coli* as substrate.

Fig. 2. Standard curves derived from curves in Fig. 1.

Estimation of bacteriolytic activity. Bacteriolytic activity is estimated by measuring the percentage lysis of a standard suspension of autoclaved *Bact. coli* by the lytic preparation in 15 min. under defined conditions. Percentage lysis is translated into bacteriolytic units (Lyt. units) by means of a standard curve. The bacteriolytic unit is defined as that amount of lysin which under the conditions described will produce 30 % lysis of a standard suspension of *Bact. coli* in 15 min.

The standard suspension of *Bact. coli* is made from twice-washed organisms, resuspended in phosphate buffer pH 8·0, and autoclaved at 120° for 20 min. After standing overnight at 4° the concentration of the suspension is adjusted so as to have the same optical density as double-strength Thompson's (1946) inorganic grey solution, as compared in a visual colorimeter with a tricolour green filter in the eyepiece. This is equivalent to a 0·04 % (dry wt./v) suspension of the organism. Freshly autoclaved *Bact. coli* is more readily lysed by the streptomyces preparation and by crude and crystalline trypsin than suspensions that have been kept at 4° for some time. After 1 hr. at 4° the lysability of the suspension becomes constant and remains so for at least 3 weeks. Therefore autoclaved suspensions of *Bact. coli* are always kept overnight at 4° before being used.

When dense bacterial suspensions, such as those used here, are compared with inorganic grey solution in a visual colorimeter in such a way that the light path (S) through the standard is kept constant while the length of the light path (U) through the suspension is varied then the ratio S/U will be a linear function of the density of the suspension. If U is kept constant and S is varied then the relationship between S/U and the density of the suspension is not linear (van Heyningen, unpublished work). Our readings of bacterial density were made in a visual colorimeter with double-strength inorganic grey solution as standard at a constant depth. The ratio S/U is a direct measure of the relative concentration of bacteria. More elaborate apparatus, such as is used by Welsch (1947), is unnecessary.

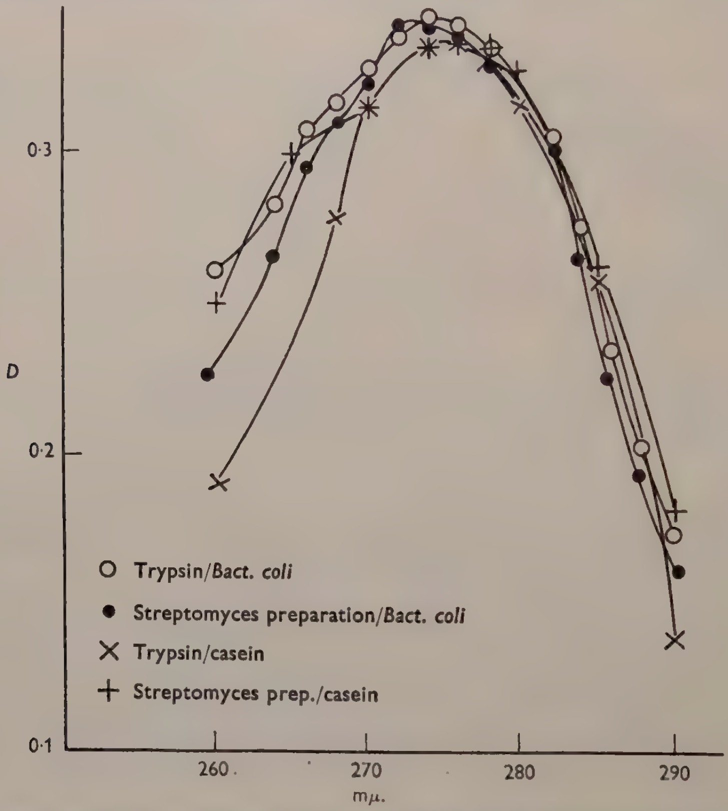

Fig. 3. Ultra-violet absorption spectra of trichloroacetic acid-soluble material liberated by crystalline trypsin from autoclaved *Bact. coli* and casein, and by the streptomyces preparation from the same substrates.

To measure bacteriolytic activity 1 ml. of a solution of the lytic agent in 0·67 M phosphate buffer pH 8·0 is mixed with 4 ml. of the autoclaved *Bact. coli* suspension previously warmed. The turbidities of the mixture at 0 and 15 min. are compared and the percentage lysis is calculated from the expression $100-100\frac{U_{0\text{ min.}}}{U_{15\text{ min.}}}$. Activity and standard curves are prepared in the same way as those for the estimation of proteolytic activity. These are shown in Figs. 4 and 5. Samples causing more than 50 % lysis are diluted.

Other determinations. Viable counts of bacteria were made by the method of Tai & van Heyningen (1950). Nitrogen was determined by the micro-Kjeldahl method. Values of pH were determined electrometrically using a glass electrode.

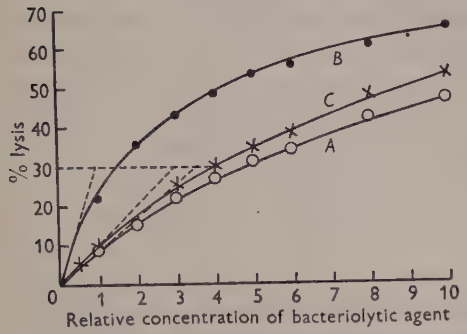

Fig. 4

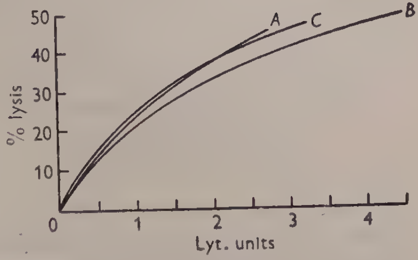

Fig. 5

Fig. 4. Bacteriolytic activity curves for the streptomyces preparation (*A*), crude trypsin (*B*), and crystalline trypsin (*C*), acting on autoclaved *Bact. coli* cells.

Fig. 5. Standard curves derived from curves in Fig. 4.

RESULTS

Adsorption of bacteriolytic and proteolytic enzymes by autoclaved Bact. coli

Autoclaved *Bact. coli* cells adsorb the bacteriolytic and proteolytic enzymes of the *Streptomyces* strain used from solution; living cells of *Bact. coli* do not adsorb these enzymes.

A 0·32 % (dry wt./v) suspension of *Bact. coli* was made in solutions of the streptomyces preparation in phosphate buffer pH 8. The suspensions were stood in ice for 30 min., the organisms were spun down, and the bacteriolytic activity (Lyt. units) and caseinolytic activity (Cas. units) of the supernatant fluids determined and compared with the values of the preparation before treatment with *Bact. coli*. About 60–70 % of the Lyt. units were adsorbed and 20–30 % of the Cas. units. It was thus obvious that the bacteriolytic enzyme(s) was not identical with the caseinolytic enzyme(s).

The presence of salts (e.g. 0·67 M phosphate buffer) inhibited the adsorption of Lyt. and Cas. units, particularly the latter. When the adsorption of enzymic activity was done in the presence of distilled water, rather than in the buffer usually used for adsorption experiments, the adsorption of Lyt. units amounted to about 85 % and that of Cas. units to about 75 %. Welsch (1947) found that salts inhibited the lysis of bacteria by his streptomyces preparation; this is possibly due to inhibition of adsorption of bacteriolytic enzymes.

When similar experiments were done with crude trypsin it was found that 60–70 % of the Lyt. units were adsorbed and 40–45 % of the Cas. units. The bacteriolytic activity of crude trypsin is also not solely due to its caseinolytic activity. When pure, crystalline trypsin was used the adsorption of bacteriolytic and caseinolytic activity was the same, viz. *c.* 60 %. Typical results are shown in Table 1, where the figures represent means of several determinations and have been brought to the nearest round numbers.

Table 1. *Adsorption by autoclaved* Bact. coli *cells of bacteriolytic* (*Lyt.*) *enzymes and proteolytic enzymes* (*Cas., casein substrate*) *from solutions of streptomyces preparation, crude trypsin and crystalline trypsin*

	Adsorption of enzymic activity (%)	
	Lyt. units	Cas. units
Streptomyces preparation	79	23
Crude trypsin	66	45
Crystalline trypsin	59	58

Although the bacteriolytic activity of the streptomyces preparation, and of the crude trypsin, could not be ascribed solely to their caseinolytic activities it could not be concluded that enzymes other than proteolytic enzymes were responsible for the bacteriolysis. It was quite possible that there might be proteolytic enzymes which were unable to digest casein but which were nevertheless able to lyse the killed organisms. Therefore the adsorption of enzymes capable of digesting the protein(s) present in autoclaved *Bact. coli* was studied. The concentrations of Lyt. and Col. units of solutions of the streptomyces preparation, crude trypsin and crystalline trypsin were determined before and after treatment of the enzyme solutions with autoclaved *Bact. coli*. Typical results of such experiments are shown in Table 2.

Table 2. *Adsorption by autoclaved* Bact. coli *cells of bacteriolytic* (*Lyt.*) *enzymes and proteolytic enzymes* (*Col.*, Bact. coli *protein substrate*) *from solutions of streptomyces preparation, crude trypsin and crystalline trypsin*

	Adsorption of enzymic activity (%)	
	Lyt. units	Col. units
Streptomyces preparation	63	59
Crude trypsin	59	57
Crystalline trypsin	36	34

These results strongly suggest that the lysis of killed *Bact. coli* by the streptomyces preparation and by crude and crystalline trypsin can be accounted for by the proteolytic activity of these preparations. Proteolytic enzymes are adsorbed before bacteriolysis, and during bacteriolysis the bacterial proteins are digested. In the case of crystalline trypsin the bacteriolytic activity is apparently due to the caseinolytic activity. Both the streptomyces preparation and the crude trypsin contained at least two proteolytic enzymes, one caseinolytic and the other capable of digesting the protein(s) of *Bact. coli*. The caseinolytic enzyme in the crude trypsin preparation is probably the same enzyme as that in the crystalline trypsin preparation, which is bacteriolytic. Whether the caseinolytic enzyme present in the streptomyces preparation is bacteriolytic remains to be seen.

These experiments show that if the streptomyces preparations contained no other enzymes besides those capable of digesting the protein in autoclaved *Bact. coli* they would be capable of bacteriolysis. They do not necessarily prove that other, non-proteolytic enzymes are not involved in bacteriolysis. The streptomyces preparation apparently did not contain any enzymes capable of breaking down the polysaccharides in *Bact. coli.* A suspension (0·04 % dry wt./v) of autoclaved *Bact. coli* was digested at 37° with the streptomyces preparation and the amount of total $(NH_4)_2SO_4$-precipitable polysaccharide determined at intervals by the method of Pirie (1939). After 2 hr. the organisms were practically completely lysed, but there was no significant diminution in the amount of $(NH_4)_2SO_4$-precipitable polysaccharide. Results of a typical experiment are shown in Table 3.

Table 3. *Lysis and proteolysis of autoclaved* Bact. coli *by the streptomyces preparation, showing apparent absence of polysaccharases*

Time (min.)	Lysis (%)	Increase in D_{280} of TCA* filtrates	$(NH_4)_2SO_4$-precipitable polysaccharide (μg./ml.)
0	—	—	17·4
10	30·7	0·025	16·7
20	44·3	0·062	17·5
40	61·7	0·072	17·2
60	71·7	0·080	16·8
120	84·5	0·100	16·0

* TCA = trichloroacetic acid.

Death of the organism as a prerequisite of bacteriolysis

The resistance of living tissues to the action of proteolytic enzymes is a problem that has fascinated biologists for a long time (see Northrop, Kunitz & Herriott, 1948). Some living micro-organisms are lysed by non-proteolytic enzymes like lysozyme, but living *Bact. coli* are apparently not lysed by the streptomyces preparation. We have studied the lysability of *Bact. coli* killed in a number of ways, but we have not been able to throw any light on the problem.

Lysis of heat-killed organisms. Bact. coli suspensions were subjected to various temperatures for various times, and the following observations were made: (*a*) the viable count of the organisms; (*b*) the degree of lysis by the streptomyces preparation and by crude trypsin; (*c*) the degree of adsorption of the bacteriolytic activity of these preparations. Fig. 6 is typical of the results obtained. Similar curves were obtained when the organisms were heated at the temperatures shown for 40, 70 and 120 min., and when crude trypsin was used instead of the streptomyces preparation.

At *c.* 55° all the organisms were killed, but there was no appreciable lysis in 15 min., and *c.* 25 % of the maximum adsorbable bacteriolytic activity was adsorbed. Only at 70° did the organisms become markedly lysable.

Although organisms killed by heating at 55° do not appear to be lysed in

15 min. they are nevertheless lysed when exposed to the lytic agent for 3 hr. Table 4 shows the lysis of organisms killed at 55° by the streptomyces preparation and by crude trypsin. The control experiment, in which the organisms were suspended in phosphate buffer alone, shows that appreciable autolysis takes place during the experimental period. Presumably the marked lysis of organisms which had been heated above 70° was due to the denaturation of bacterial protein.

Table 4. *Autolysis, and lysis by the streptomyces preparation and crude trypsin, of* Bact. coli *cells killed by heating at 55° for 1 hr.*

	Lysis at 37° (%) after periods of time shown		
Lytic agent	**15 min.**	**1 hr.**	**3 hr.**
Phosphate buffer	4·2	10·5	16·8
Streptomyces preparation	8·7	20·1	43·8
Crude trypsin	7·6	16·6	35·4

Lysis of organisms killed by other means. A study was made of the lysis of suspensions of *Bact. coli* killed by the following means: (*a*) standing for 12 days in distilled water; (*b*) treatment with a 1 mg./ml. concentration of polymyxin E (Burroughs Wellcome Ltd. 'Aerosporin'); (*c*) treatment with a 3 mg./ml. concentration of polymyxin. When *Bact. coli* was suspended in distilled water for 12 days 90·1 % of the organisms died. Practically all the organisms were killed after exposure to 1 mg. polymyxin/ml. for 3 hr.

The results for the lysis of the organisms killed by these means are given in Table 5.

Table 5. *Lysis by autolytic enzymes, by the streptomyces preparation, and by crude trypsin of 'living'* Bact. coli, *and of organisms killed by various means*

		Lysis at 37° (%) after		
Lytic agent	**Treatment of *Bact. coli***	**15 min.**	**1 hr.**	**3 hr.**
Phosphate buffer	'Living' organisms	4·9	11·0	23·4
Streptomyces preparation		6·7	13·9	26·7
Crude trypsin		8·1	15·5	25·3
Phosphate buffer	Organisms dead after standing in distilled water for 12 days	11·4	22·0	37·5
Streptomyces preparation		18·5	33·5	45·5
Crude trypsin		18·8	30·5	42·6
Phosphate buffer	Organisms killed with polymyxin 1 mg./ml. for 3 hr.	12·0	17·8	25·4
Streptomyces preparation		26·3	35·9	44·3
Crude trypsin		21·7	31·2	39·6
Phosphate buffer	Organisms killed with polymyxin 3 mg./ml. for 3 hr.	11·6	13·6	17·8
Streptomyces preparation		22·2	38·4	48·3
Crude trypsin		17·8	33·0	43·8
Phosphate buffer	Organisms autoclaved at 120° for 20 min.	0	3·0	7·1
Streptomyces preparation		34·4	64·0	Out of range
Crude trypsin		41·5	71·1	Out of range

All the lysis of living organisms (bearing in mind that a suspension of living organisms must contain many dead organisms) is due to autolysis; but organisms which have been killed by comparatively gentle means are to some extent lysable by the streptomyces preparation and by crude trypsin. The change that takes place when an organism dies, rendering it susceptible to lysis, is not known. All we do know is that dead organisms adsorb more of the lytic agent than living organisms. This is shown in Table 6.

Table 6. *Adsorption of bacteriolytic activity from the streptomyces preparation by 'living'* Bact. coli, *and by organisms killed by various means*

Treatment of *Bact. coli*	Adsorption of bacteriolytic activity from streptomyces preparation (%)
Living	10·4
Dead after standing in distilled water for 12 days	16·7
Killed by heating at 55° for 1 hr.	26·5
Killed by treatment with 1 mg. polymyxin/ml. for 3 hr.	34·5
Autoclaved at 120° for 20 min.	69·8

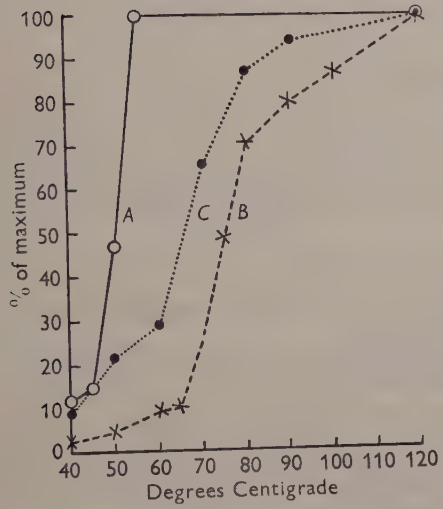

Fig. 6

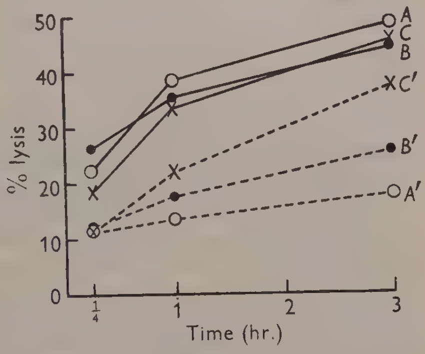

Fig. 7

Fig. 6. Effect of heating for 10 min. on: (*A*) mortality of *Bact. coli*; (*B*) degree of lysis of *Bact. coli*; (*C*) the degree of adsorption of bacteriolytic activity. Bacteriolytic agent, the streptomyces preparation. Values of ordinate are given as percentage of maximum attainable at 120°.

Fig. 7. Autolysis and lysis by the streptomyces preparation of *Bact. coli* killed by: (*A*) polymyxin, 3 mg./ml.; (*B*) polymyxin 1 mg./ml.; (*C*) standing in distilled water for 12 days. *A*, *B*, *C*, lysis; *A′*, *B′*, *C′*, autolysis.

Autolysis

A re-examination of the data in Table 5 might throw some light on the autolysis of *Bact. coli*. 'Living' organisms autolyse to some extent; autoclaved organisms hardly autolyse at all, presumably because the autolytic enzymes have been destroyed. Organisms killed by more gentle means autolyse more than 'living' organisms. Pulvertaft & Lumb (1948) observed that organisms killed with minimal concentrations of antiseptics undergo extensive autolysis. Organisms that have been killed with 3 mg. polymyxin/ml. autolyse less than organisms that have been killed with 1 mg. polymyxin/ml., although the degree of lysis by the streptomyces preparation is about the same in both cases. This suggests that the higher concentration of polymyxin has inhibited the autolytic enzymes. The degree of lysis by the streptomyces preparation of organisms killed by standing in distilled water for 12 days, or by treatment with 3 or 1 mg. polymyxin/ml. is much the same irrespective of the autolysis of these organisms (see Fig. 7). This suggests that the effects of the streptomyces enzymes and the autolytic enzymes are not additive, and therefore that the streptomyces enzymes and the autolytic enzymes act upon different substrates. Possibly proteolytic enzymes play only a small part in autolysis.

We are grateful to Prof. Sir Howard Florey, F.R.S., for his encouragement and interest in this work, to Dr G. V. R. Born for collaboration in certain experiments, to Dr Kathleen Crawford for identifying the organism, and to Miss Megan Lancaster for technical assistance. One of us (T.Y.T.) held a scholarship from the Chinese Government and grants from the University of Oxford and the British Council which are gratefully acknowledged.

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Virus-like Bodies in the Blood of the House Cricket

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SUMMARY: Virus-like structures were accidentally discovered by electron-microscopy in the blood of adult house crickets which had no external signs of disease.

During an examination by electron-microscopy of haemolymph clots of insects, granular bodies were found by chance in preparations from the house cricket (*Gryllulus domesticus*). These structures are briefly described in the following note.

MATERIAL AND METHODS

The preparations consisted of thin films of haemolymph from the antennae or legs of adult specimens of apparently disease-free crickets. A small drop of haemolymph was spread between slide and cover-slip previously coated with 0·15 % (w/v) formvar solution in ethylene dichloride or 0·5 % (w/v) parlodion in amyl acetate, and fixed by immersion in a 2 % (w/v) solution of osmic acid. Small areas of plastic film supporting the blood preparations were then cut from the surrounding film with a pair of sharp scissors, peeled away from the glass and transferred under water on to standard stainless steel screens for electron-microscopy. The covered screens were then lifted from the water, drained, dried and examined or photographed with an R.C.A., type E.M.U. electron microscope.

Twenty-five adult crickets belonging to the breeding stock of the Department of Biochemistry were used and about two hundred screens examined. As the findings were made after the insect specimens had been discarded, no anatomical study of the tissues could be performed.

RESULTS

On examining these preparations under the electron microscope, minute granules, either in high concentration (fig. 1, see Pl. 1) or scattered at random (figs. 3–5, 10 and 11) were found. Most of them appeared to be oval in shape with a definite internal organization, consisting of a central dense rod-shaped structure, surrounded by a relatively transparent area (figs. 2, 15). Circular areas of high density were occasionally visible either at both ends of the rod (figs. 3, 4, 10 and 11) or aligned within it (fig. 16). Less frequently, forms were found in which the central rod was not visible (figs. 6, 11), or only faintly outlined (figs. 7, 9, 10, 12 and 13), with two spherical polar zones of higher density distinctly apparent. These dense polar areas were sometimes the only internal structures seen in the egg-shaped transparent body (fig. 12). As seen in fig. 14, the dense internal rod was not always located at the centre of the granule: the clear area then appeared on one side of it. This fact, together with large variations in diameter of the clear oval area, suggests that the latter structure may have some plasticity around the relatively more rigid central rod-like structure. Figs. 8 and 11 show that this rod may, however, bend, giving the granule a more spherical shape. From measurements on electron micrographs of three hundred internal rods, their average size appeared to be approximately 223 × 57 mμ.

These bodies were found in the haemolymph of eight out of twenty-five adult crickets. The frequency of occurrence of these structures was probably higher, but owing to their scarcity in some preparations, they might have been overlooked in the preparations from the other insects.

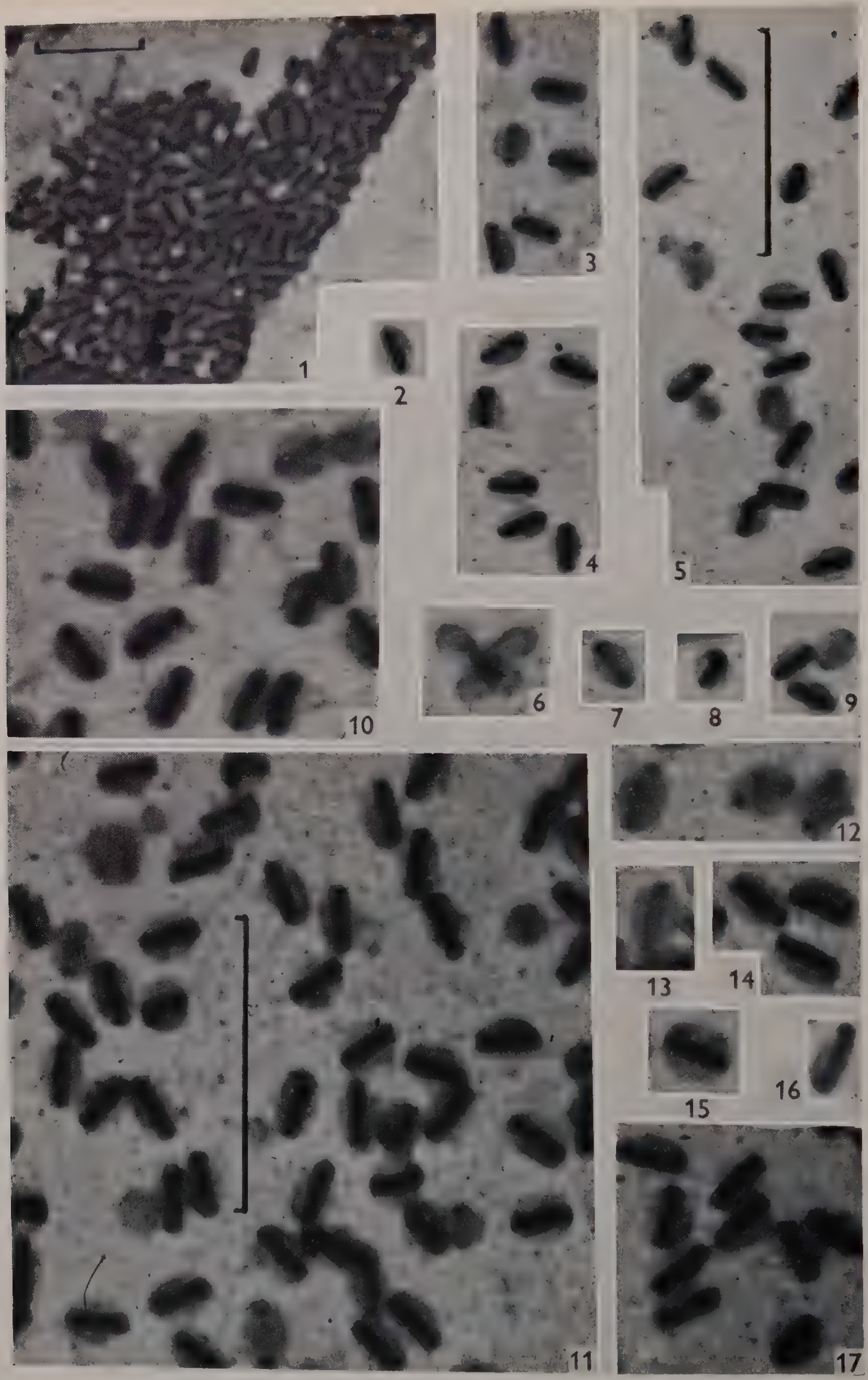
Most of the haemocytes visible in the films of coagulated haemolymph were disintegrated and their cytoplasmic constituents (granules, mitochondria, endoplasmic particles) were distributed in the surrounding plasma coagulum. The presence of virus-like bodies scattered among the cytoplasmic structures suggests that they, too, were originally inside the haemocytes, although they appear free in the plates. The thickness of the remaining intact haemocytes did not allow critical observation of the minute structures within their cytoplasm.

DISCUSSION

Granules showing some similarity of structure with those described in the present note have been recognized as viruses and etiological agents of granulosis in insects (Paillot, 1934) and demonstrated by means of the electron microscope by Bergold (1948), Steinhaus, Hughes & Wasser (1949), and Steinhaus & Thompson (1949). Bergold (1948) observed in the cytoplasm of blood cells of infected caterpillars of the pine shoot roller, *Cacoecia murinana*, Hb., vesicular structures containing large numbers of egg-shaped particles having a size of 360 × 230 mμ. On treatment with sodium carbonate solution, these particles (capsules) released rod-like structures, 262 × 50·6 mμ. in size, and resembling the virus particles found in polyhedroses.

Under the electron microscope Steinhaus *et al.* (1949) observed opaque oval granules with no significant internal structure, and having a size of approximately 250 × 400 mμ. in suspensions of infected tissues of the variegated cutworm, *Peridroma margaritosa* (Haw). In preparations of fat tissue from infected cutworms suspended in sodium carbonate, the granules lose some of their dense aspect, and a relatively clear rod-shaped area or cavity may be seen more or less centrally located along the long axis of the granule. Rod-shaped particles of about the same dimensions as the cavities and representing probably the virus itself can be seen lying among the granules. Similar ovoid granules, of about 350 × 500 mμ. in size, and enclosing rod-shaped virus particles, were recently demonstrated by Steinhaus & Thompson (1949) in diseased tissues of the Buckeye caterpillar, *Junonia coenia*.

There are important differences between the morphological features of the granules described in the present note and those studied by Bergold and by Steinhaus and his co-workers. The granules described by these workers are considerably larger, and consist of a dense solid substance, impermeable to electrons, in which the virus particles and other internal structures can only



Figs. 1–17

CH. GRÉGOIRE—VIRUS-LIKE BODIES IN THE HOUSE CRICKET. PLATE 1 be seen after treatment with alkali. Bergold's free virus particles have approximately the same size as the rod-shaped structures described here.

In the present material, the internal structures of the bodies were visible within their transparent envelope or sac without any treatment other than fixation with osmic acid. No liberation of dense rod-shaped particles from the sacs was observed. In addition, peculiarities of structure (circular areas of high density within some internal rods, and forms with two dense spherical polar areas visible within the envelope at the location of the rods) not mentioned in the descriptions of Bergold or of Steinhaus *et al.* suggest that the present bodies belong to a different type of organism with a complex internal organization. More detailed studies of these virus-like bodies and of various organs and tissues of insects from the same stock will show whether *Gryllulus domesticus* is a host species for a distinct disease of the group 'granuloses' or a 'healthy carrier' of a type of latent insect virus distinct from both the polyhedral and granular diseases.

I wish to express my thanks to Mr G. R. Wyatt, Virus Research Unit, Molteno Institute, Cambridge University, for several helpful suggestions while reading the manuscript.

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EXPLANATION OF PLATE

Electron micrographs of bodies in thin films of haemolymph, fixed by immersion in 2% (w/v) osmic acid solutions, from adult specimens of *Gryllulus domesticus*. Magnification: the scales correspond to 1μ. Figs. 2–9 have the same scale as fig. 5. Figs. 10–17 have the same scale as fig. 11.

Fig. 1. Agglutinated bodies in the plasma coagulum.

Figs. 2 and 15. Typical oval-shaped bodies.

Figs. 3–5, 10, 11 and 17. Bodies of different types described in the note, scattered in the plasma coagulum.

Fig. 6. A group of transparent envelopes or sacs without rod-shaped internal structure.

Figs. 7, 9, 12 and 13. Forms with dense circular polar areas, and in which the central rod-shaped structure is not visible or only faintly outlined.

Figs. 9, 14 and 16. Bodies with clear envelopes or sacs mostly located on one side of the internal rod.

Fig. 8. Body with a curved internal rod. A similar body is seen in fig. 11.

Fig. 16. Possible alignment of four dense areas within a rod-shaped internal structure.

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The Vitreoscillaceae: A Family of Colourless, Gliding, Filamentous Organisms

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SUMMARY: A new family, the Vitreoscillaceae, is proposed for a group of microorganisms which are characterized by their colourless, gliding trichomes. The family has certain affinities with myxobacteria and still more with colourless myxophyceae, especially with the Beggiatoaceae.

Two genera, *Vitreoscilla* and *Microscilla* and seven species are described, three species tentatively referred to *Vitreoscilla*, two to *Microscilla*, and two other genera are provisionally named.

Colourless trichomes with a gliding movement are so common that it would be surprising if they had been overlooked, but descriptions are in many instances so vague that the species described cannot now be identified.

Cohn (1870–1, p. 121) mentions 'creeping, short, Oscillaria-like filaments, composed of up to eight cylindrical sections, which exhibited a peculiar slow, gliding movement', which probably belonged to the group with which this paper deals. Van Tieghem (1880, p. 177) observed colourless Myxophyceae, perhaps *Spirulina*. The organism described by Winogradsky (1888, p. 23) as *Beggiatoa minima* i.e. forms not more than 1 μ. in width, does not, according to Lauterborn (1915, p. 419), belong to the sulphur bacteria and may well have been a member of the group here described. The flexible organisms described by Dobell (1908, 1912) do not appear to be related, since the author ascribes flagella to them. *Spirulina albida* (Kolkwitz, 1909, p. 137) is the first gliding, filamentous, heterotrophic organism which can be identified, while in a number of forms assigned to the genus *Oscillatoria* by various authors, lack of chlorophyll is not established beyond doubt (Pringsheim, 1949, p. 62).

A few myxobacteria (Garnjobst, 1945; Nigrelli & Hutner, 1945) may be mentioned here. While the majority are not filamentous, but rod-shaped, those myxobacteria which form longer trichomes composed of several cells, are definitely colourless, gliding and filamentous. Their affinity with the organisms here described has therefore to be discussed in this paper. For lack of information I am unable to establish the taxonomic position of Soriano's (1947) *Flexibacter* which may either belong to the Vitreoscillaceae or more probably to the Myxobacteria.

Achroonema spiroideum, described by Skuja (1948), may also be related to the Vitreoscillaceae, although it is assigned by the author to the Oscillospiraceae, Peshkoff (1940). *Oscillospira* is, however, a true bacterium with flagella, showing a very different organization from that of the family here described (Pringsheim, 1949, p. 74). This taxonomic discrepancy has also struck Brunel (1949) who found a form similar to Skuja's, to which he even gives the same name, although the latter is 0·3–0·5 μ. wide, and Brunel's 1–1·5 μ. This difference is not negligible since there may be more than one such organism. Experience shows that the Vitreoscillaceae comprise a large group of related forms. Skuja's taxonomic proposals are evidently influenced by Peshkoff's rather confused ideas (cf. also Bergey's Manual, 1948, p. 1002), and not based on personal knowledge of *Oscillospira* and *Caryophanon* which in no way resemble gliding organisms like *Vitreoscilla* to which *Achroonema* possibly belongs.

In 1947 I read a paper on this subject to the Society for General Microbiology and gave a demonstration of six species of *Vitreoscilla* in pure culture, illustrated by drawings, photographs and microscopical preparations. To change the name to *Achroonema* would thus cause confusion, especially as cultures were given to various laboratories, and an article, including descriptions of species, was published in *Bacteriological Reviews* (Pringsheim, 1949, p. 70).

The gliding movement

The word gliding is preferable to creeping or crawling which both suggest the movements of certain vertebrates, with which the gliding of these lowly organisms has no resemblance. Gliding movement will be called in this paper a translocation along solid bodies or the interface between water and air, during which no wriggling, contraction or peristaltic alterations are visible, the change of shape being restricted to bending. Whereas *Peranema*, a colourless, zootrophic eugleninean, generally moves along the surface of the water, myxophyceae do not seem to be able to do so, except when they support one another (Engelmann, 1879), and this appears to be true also of the Vitreoscillaceae. The gliding movements are not always regular, but intermittent and hesitant, with frequent changes of direction, as in the unicellular Myxophyceae and Rhodophyceae, Myxobacteria and Vitreoscillaceae (Pringsheim, 1949). While the suggestion that this kind of movement is caused by wave-like contractions, which was first put forward by Engelmann (1879, p. 55), then by Schmid (1918, 1919), and supported by experiments and observations by Ullrich (1926, 1929), has been generally accepted for filamentous Myxophyceae and Beggiatoaceae, it cannot easily be applied to unicellular organisms, and is very doubtful in the case of Myxobacteria, hormogonia of Myxophyceae, and Vitreoscillaceae. There is not even a hypothesis to explain the gliding of these organisms (Pringsheim, 1949, p. 65), except those based on the formation of mucilage. This was studied by Hansgirg (1887) who found that particles added to a preparation were carried in a definite direction, a phenomenon more fully investigated by Correns (1897) and by Kolkwitz (1897), and recently by Niklitschek (1934). Fechner (1915) explained the movement of *Oscillatoria* by an anisotropic swelling of the mucilage in a direction oblique to the axis, a hypothesis not widely accepted. In unicellular forms and those with short trichomes, however, unequal secretion of mucilage at the two poles might well provide the clue in those instances where waves of contraction cannot be accepted as an explanation. A polar exudation of mucilage as a method of locomotion is known in many lowly organisms.

All the gliding structures which are appreciably longer than they are wide show a marked flexibility. Probably this does not mean active bending (Correns, 1897) but only a lack of rigidity, so that small obstacles to their progress may cause a distortion (Pringsheim, 1949, p. 65). This pliability is probably due to the absence of a cell wall, although in some cases it is the connecting link between the cells rather than the cells themselves which is least resistant to bending.

THE VITREOSCILLACEAE

The organisms described below bear so close a resemblance to certain members of the Oscillatoriaceae that they may be considered to be colourless Myxophyceae. This is borne out by the following features which they have in common with the latter: the lack of true nuclei, filamentous habit, gliding locomotion, reproduction by hormogonia. Some species might even be mistaken for faintly coloured species of *Oscillatoria* or sulphur-free *Beggiatoa*. Others differ in having short trichomes, but these are also found among true, pigmented Oscillatoriaceae.

The number of colourless, gliding, filamentous organisms seems to be considerable. Those described by Soriano (1947) differ from those I have found, and all those I have isolated in pure culture represent distinct species. It will take time to describe the group more completely; at present only those which have been grown in bacteria-free culture, and a few readily recognizable forms, can properly be named.

Localities where members of the Vitreoscillaceae can usually be found are the following: floating masses of pigmented Myxophyceae, water with decaying plant material, mucilage formed by other organisms, water-flowers of Chroococcaceae, cow dung, and manured soil.

The members of the Vitreoscillaceae differ from one another mainly in the width of their trichomes, and in the ratio of the width to the length of their cells. Some have barrel-shaped cells, divided by constrictions, others a cylindrical shape of even width. They also vary in their degree of flexibility and in the celerity of their movements. The latter two characters determine the type of movement and the shape of the trichomes. The more rigid forms move mainly in a longitudinal direction with only their free ends rotating in a narrow angled cone, in a way similar to that of the wider species of *Oscillatoria*. The pliable species appear as curved, irregularly wavy threads which change continually in shape and direction of movement.

The more moniliform species have a marked tendency to break up into short, hormogonium-like lengths whereas the cylindrical ones grow out into filaments of almost unrestricted length, as also occurs in certain species of *Oscillatoria* and *Beggiatoa*.

Some species of Vitreoscillaceae might be regarded as unpigmented species of the genus *Oscillatoria* in the same way as *Spirulina albida* (Pringsheim, 1949, p. 62) is assigned to its genus despite its lack of pigmentation. Those which resemble *Beggiatoa* differ from that genus in their inability to form sulphur from hydrogen sulphide and may even occur among *Beggiatoa* trichomes, which are themselves full of sulphur, without containing any. In view of the number of species and the gradual transition between long filamentous forms and those which retain a hormogonium-like state, which is distinctly different from the general condition in *Oscillatoria*, the group was given a new name, the Vitreoscillaceae with *Vitreoscilla beggiatoides* n.sp. as the type. They could perhaps be included in Soriano's (1947) Flexibacteriales if this order were revised (Pringsheim, 1949, p. 85).

The Vitreoscillaceae are provisionally divided into four genera: *Vitreoscilla*, *Microscilla*, *Bactoscilla* and *Flexoscilla*. In *Vitreoscilla* the trichomes are composed of well-marked cells, cylindrical like those of *Beggiatoa alba*, or barrel-shaped like those of *Beg. mirabilis*. *Microscilla* possesses narrower filaments, in which cells cannot be discerned by optical means, although this difference may prove to be only one of size. Soriano's (1947) genus *Flexibacter* may possibly overlap with *Microscilla*, but my forms are definitely not bacteria. *Bactoscilla* is peculiar in that its trichomes are composed of rod-shaped sections connected by pliable links. *Flexoscilla* represents slender rods becoming narrower at each end. Pure cultures were obtained only of species of *Vitreoscilla* and of *Microscilla*.

VITREOSCILLA n.gen.

Species grown in pure culture

Vitreoscilla beggiatoides n.sp. This species was first isolated in 1941 from a ditch on Coldham Common, Cambridge, with decaying water weeds, where it occurred together with *Sphaerotilus discophorus*, *Beggiatoa alba*, and many bacteria. After plating in various ways had failed, floating trichomes were washed five times with the help of capillary pipettes and transferred singly to agar plates. In one Petri dish, with Heyden agar, after a week the entire surface was covered with colourless curling filaments, from which a new plate was inoculated by streaking. Transfers from this remained free from bacteria. The observation of isolated nests of development at a distance from the main growth confirmed the identity of the organism, which can glide over the agar. The ease with which bacteria-free cultures were obtained is due to this ability to glide.

The trichomes of this species are about 2μ. wide, sometimes more or less, and may grow so long that they cannot be measured with certainty, being curved and more than 150μ. in length. They often show shallow constrictions at distances of $4–5\mu$. or more apart, evidently preformed places of division. The smallest trichomes are rarely shorter than 10μ. and composed of two sections. All are rounded at the tip (Fig. 1). When cultivated in liquid media the trichomes may grow out into perfectly cylindrical filaments of almost unrestricted length before they show periodical constrictions and break up into short lengths, a kind of ill-defined hormogonia. On agar such long filaments are not formed. While young, healthy, growing trichomes scarcely show any inner structures except the septa, older ones contain many refractive granules. By staining with Robinow's technique (i.e. HCl-hydrolysis and Giemsa), nuclear bodies, which also give the Feulgen reaction, can be demonstrated.

The organism resembles a sulphur-free *Beggiatoa* and, like it, is motile while in a healthy condition. Under a cover-slip it ceases gliding and can then scarcely be distinguished from a sheathless *Sphaerotilus* filament. In hanging drops the movement begins after a few minutes during which the trichomes fasten themselves to the glass. The movement is a slow but perceptible steady advance in the direction of the axis. Direction may be reversed without visible cause. When gliding trichomes are caught between particles, one end swings funnel-wise in the way known in *Beggiatoa* and *Oscillatoria*. Filaments of the same species do not seem to affect the movement in the same way as inanimate particles. The trichomes glide along each other, and on agar considerable numbers may arrange themselves in parallel. Bacteria and debris adhere to them very little; the reason is a soft mucilage of which no definite boundary can be demonstrated by indian ink.

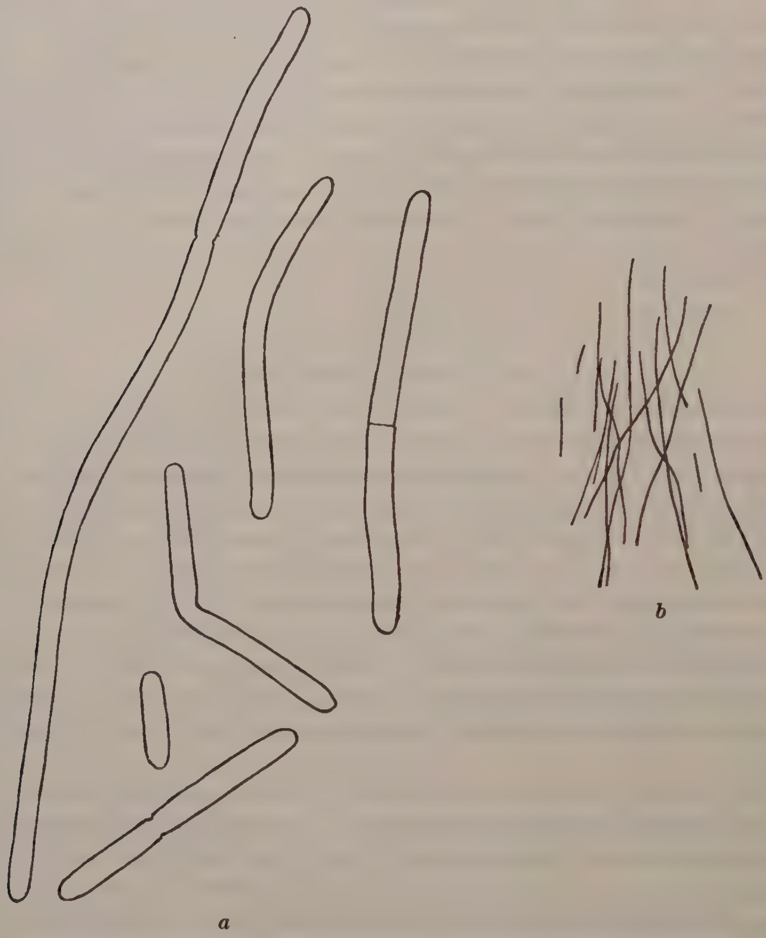


Fig. 1. *Vitreoscilla beggiatoides*. *a*. Trichomes of various lengths, all of them motile, × 1500. *b*. Bundle of trichomes from liquid medium; × 200.

The growth on agar is very characteristic; the trichomes form curls, spirals and waves (Figs. 2, 3). If the surface is not too dry they spread from the place of inoculation and colonize the whole area. In liquid media the cultures are not easy to see, owing to the low refraction of the organisms. Multiplication takes place most freely near the surface of the culture fluid adhering to the glass wall, whence small floccules can be seen to sink to the bottom at the slightest disturbance of the fluid. The best growth in a liquid occurs in the condensation water of agar slopes.

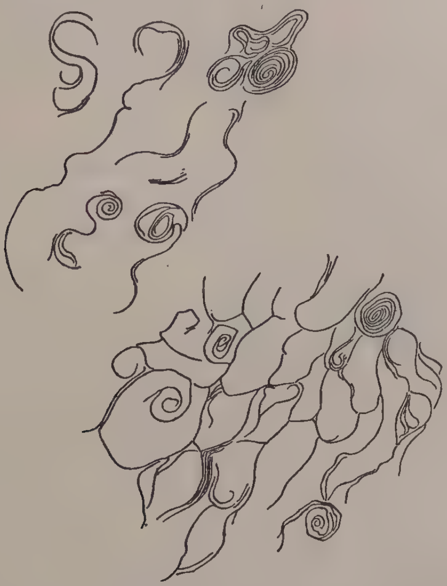
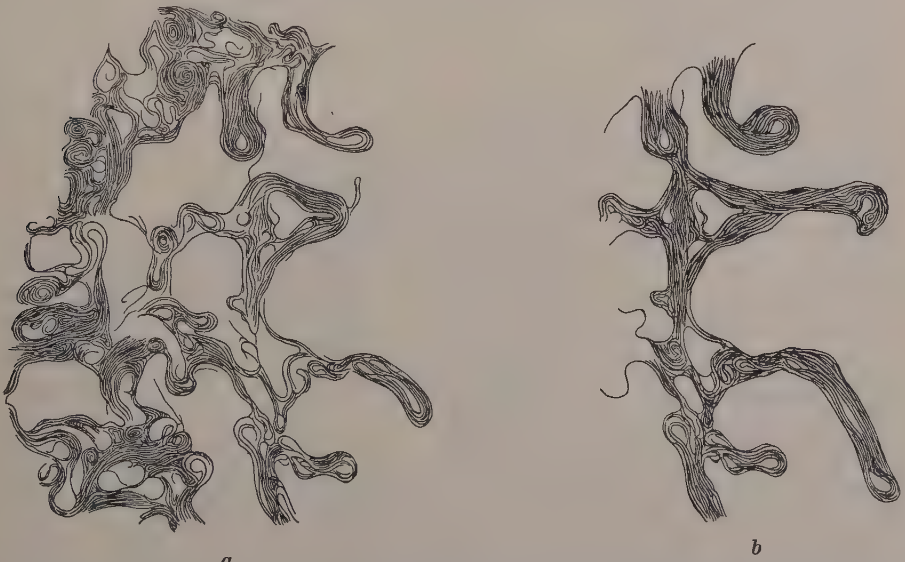


Fig. 2. *Vitreoscilla beggiatoides.* Dispersed growth with local spirals on agar with 0·2 % Heyden nutrient + dilute Benecke solution; × 50.



a *b*

Fig. 3. *Vitreoscilla beggiatoides.* *a.* Edge of large colony on Heyden agar. *b.* Seven minutes later; note change in disposition of trichomes and projections; × 150 (from photographs which, owing to the movement, were not sharp enough for reproduction).

Growth is abundant on agar media with beef-extract, yeast-extract, Difco proteose peptone (all 0·1–0·3 %) and still better in mixtures of them, if the concentration is not too high and the pH not lower than 6·5. Glucose, acetate, glycerol and starch are without marked influence at low concentrations and harmful at higher ones. On a medium with 8–10 % gelatin growth is moderate, and the spreading, compared with agar, is restricted. Some growth may occur on plain gelatin which, however, is not liquefied, so that no proteolytic enzyme appears to be produced. This is confirmed by cultures upon casein agar which does not become translucent around the growth.

Vitreoscilla beggiatoides is found, but not regularly, in localities like the original habitat, i.e. quiet waters rich in organic matter, with black mud on the bottom, which often harbour a considerable variety of Vitreoscillaceae. Most of the trichomes are attached to solid bodies, but can be detached by shaking. The free-floating trichomes do not settle quickly and can be used for isolation.

Vitreoscilla catenula n.sp. This species was observed several times in ditches between meadows in Cambridge. Attempts at isolation, however, failed until apparently the same species was found in an infusion of cow-dung from the same meadow, and was streaked out on dung agar where it developed a similar growth to that of the former species. By repeated plating bacteria-free cultures were obtained, again by making use of its gliding motility for isolation.

Trichomes of this species may be cylindrical and of almost even width; usually there are constrictions between the cells, which are then barrel-shaped. They vary more in width than the previous species, being 1·5–2μ. in diameter and generally 3–4, sometimes up to 6μ. long (Fig. 4*a–c*). The specimens first found in 1941 in material from a ditch in Cherry Hinton, Cambridge were of these dimensions, and so were those in the strain derived from cow-dung in 1945. For this reason these organisms are believed to be identical. No length of trichomes can be given; they may break up into short fragments or grow to some length.

Because of the constrictions between the cells, trichomes of *V. catenula* resemble the blue-green genus *Pseudanabaena*, especially *P. tenuis* Koppe, although there is no indication that they have originated from this slightly smaller form. The bead-like appearance of the cells is correlated with a greater tendency to break up into shorter lengths than is found in *Vitreoscilla beggatoides*. Very long filaments are rarely found in *V. catenula*. Another difference is the rounding off of the cells of the latter in old cultures, comparable to the 'involution' of bacteria and associated with a considerable increase of width.

In nutrition and the appearance of the growths upon agar and in liquid media, the two species are very similar.

Vitreoscilla filiformis n.sp. This species was first observed in November 1942 in a crude culture consisting of a grain of wheat covered with soil and water from the pond in the Cambridge Botanic Garden. The trichomes are more elastic than those of the other species, and on agar they form neatly rounded arches and loops like those of certain species of *Oscillatoria* (cf. Cataldi, 1941, Pl. II, fig. B; Pl. III, fig. A) which are not found in any of the other *Vitreoscilla* cultures. This pronounced elasticity is combined with a greater tensile strength so that very long filaments are formed, not only in liquid media but also on agar (Pl. 1, fig. 1). Shorter trichomes are, however, regularly present; both long and short ones exhibit an active gliding and swinging of free ends. The species is more slender than the two previously described, having a regular diameter of 1·2 μ.

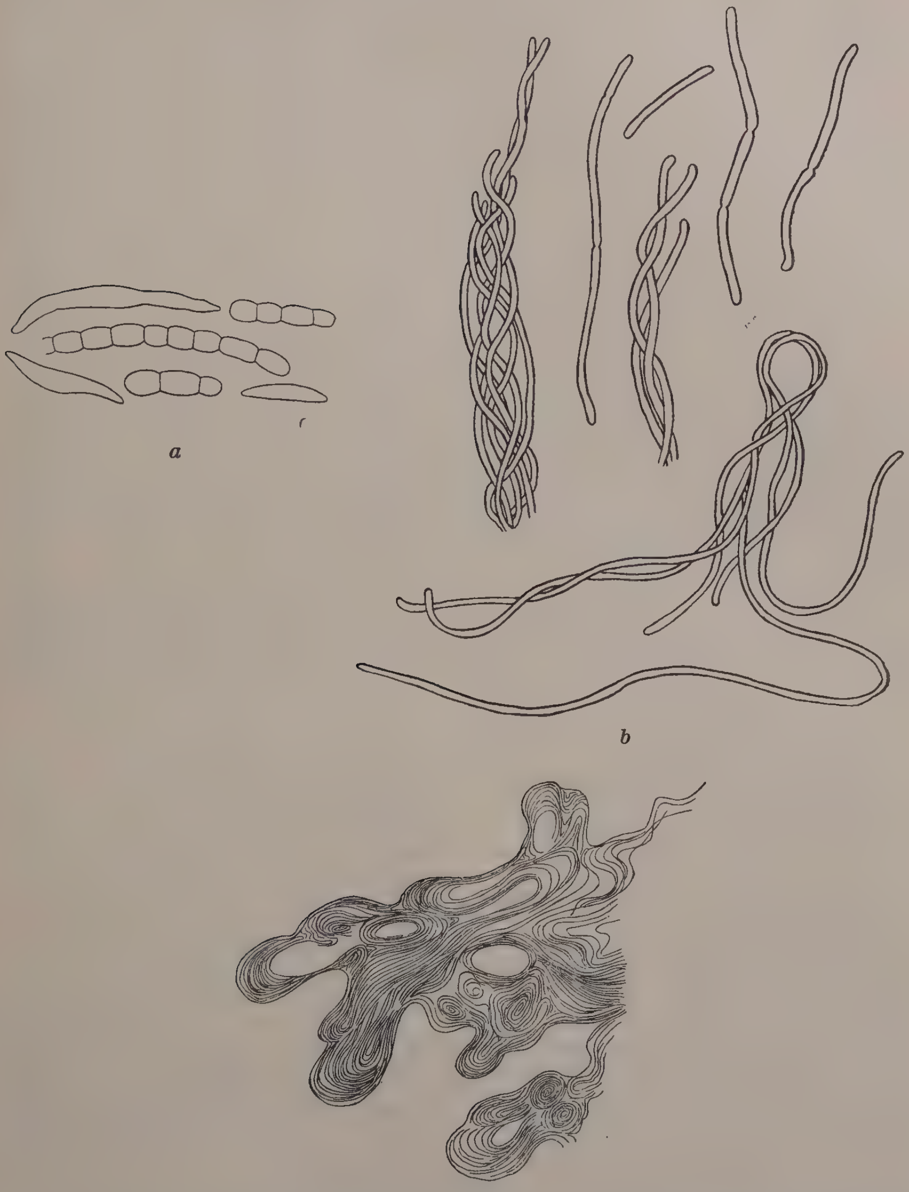


Fig. 4. *Vitreoscilla catenula*. *a*. Trichomes and involution forms, × 1500. *b*. Single trichomes and bundles; × 400. *c*. Colony on yeast extract agar; × 100.

From a non-nutrient agar plate, inoculated by Dr B. N. Singh with soil for studies on amoeba, the same or a very similar form was isolated by Dr R. Y. Stanier at Rothamsted Experimental Station (Pl. 1, fig. 2). Stanier used dung agar for the isolation which, however, was not suitable for maintenance so that the pure culture was lost, but I was given the opportunity of comparing it with my own cultures. This species was found again and grown in pure culture from cow manure streaked on 0·1 % proteose-agar. Maintenance was again difficult. No suitable medium was found other than the unreliable cow-dung agar which, because of its variable composition, makes subcultivation uncertain. This is also made difficult by the cohesion of the growth. Cultivation was possible on proteose and proteose beef-extract agar and on routine bacterial nutrient agar. (0·5 % beef-extract + 0·5 % Bactopeptone, pH 7·5). Higher concentrations and a slightly acid reaction prevented multiplication.

Apart from its smaller width *V. filiformis* differs from *V. beggiatoides* in the appearance of the colonies on agar, which are delicate and almost circular, with a slightly denser centre and fringes at the periphery, while those of the latter are curly and have a very irregular outline. In liquid media *V. filiformis* develops initially only on the bottom, growing then higher up and outward from its points of attachment, whereas *V. beggiatoides* forms cobweb-like thin films, covering the inner surface of the tube, which later become denser and form loose agglomerations.

This species can easily be confused with *Lineola longa* Pringsheim (Pringsheim, 1950). This bacterium, likewise occurring in cow-dung, forms similar colonies and, under certain circumstances, very long filaments of nearly the same width. Differences are as follows:

Lineola longa	*Vitreoscilla filiformis*
Flagellar movement	Gliding movement
Constrictions preparatory to division	Cylindrical of even width
Cells very long	Cells short
Easily breaks up into preformed rods	Tough threads; sometimes breaking up into short bits
Colonies irregular, on suitable media thicker	Colonies rounded, delicate
In liquid media motile trichomes or silky bundles	Tassel formation on glass surface
Adheres to agar surface	Comes off readily as a whole

Vitreoscilla moniliformis n.sp. The organism was found by Dr C. F. Robinow in June 1947 on a plate of 0·3 % yeast extract + 0·3 % Bactopeptone agar inoculated with cow-dung. He noticed it to be peculiar in swarming periodically to form wider and wider circular zones on the agar surface, rather as *Proteus* does. He kindly gave me cultures for further investigation.

In order to obtain pure cultures plating was repeated, and various liquid and agar media were inoculated from isolated colonies by the use of capillary pipettes. At 22·5° and 27° growth was well developed after one day. On a routine bacteriological agar circular colonies with zonal growth were formed. On neutral 0·2 % Difco yeast extract agar growth was similar but less profuse. In Bactopeptone + yeast extract and in Bactotryptone + yeast extract solutions, 0·3 % in each case, a homogeneous turbidity developed which, under a strong hand-lens, was seen to be composed of trichomes. As with many bacteria and other saprophytic micro-organisms, mixtures of yeast extract or beef extract with various peptones support growth better than either one alone.

On plates the growth of *Vitreoscilla moniliformis* can be seen at low power to be composed of relatively large, streptococcus-like chains (Pl. 2, figs. 3, 4). The trichomes in mounted preparations were found to be up to 150 μ. in length but generally only 30 μ. long. They are composed of a limited number of rod-shaped sections which are rounded at their ends and often appear to be interrupted by short gaps (Fig. 5*a*), but the trichomes move as a whole. The sausage-shaped sections are mostly 2·2–2·5 μ. wide and not very regular in form, often inflated to a diameter of 2·8–3·0 μ., sometimes little longer than they are wide, but sometimes as long as 15 μ., elongated and cylindrical. They tend to separate from one another. Units of four to five cells are often found. The point where a cell divides is marked long before by a constriction.

The movement of this big *Vitreoscilla* is never very active, as there is in the Vitreoscillaceae an inverse ratio between width and speed of movement. Only in young cultures is the movement directly perceptible under the microscope. If cultures are kept at 26° motility is most pronounced after 4–6 hr. and even then, observed in a hanging drop it is irregular and interrupted. The movement often appears as a flexion between two stiff rod-shaped cells, but even single cells, with only an indication of constriction near the middle, may be motile, so that the source of movement cannot lie in the joints between the cells. These are merely the points of least resistance, and bending seems to be due to friction (cf. Pringsheim, 1949, p. 84).

No cell-walls could be detected by normal illumination in spite of the relatively large size of the cells. By dark-ground illumination the edges of the cells are brightly illuminated, so that there is evidently a denser layer, which can also be demonstrated by staining (Pl. 2, fig. 4). By the use of hypertonic solutions, e.g. 5 % glucose or double concentrated sea water, irregular shrinking is caused without cell-walls becoming visible, so that one cannot speak of plasmolysis (cf., for example *Lineola* Pringsheim, 1950). The growth on agar is not always very characteristic and may be rather diffuse, but the edges, particularly on less rich media, are composed of tongue-like processes while rounded, twisted areas may be seen in the interior of large colonies (Fig. 5*b*, *c*). Against the light the growths are yellowish, against a dark background they show a mother-of-pearl effect. In liquid media, a marked turbidity and later a sediment, is formed. On the whole *Vitreoscilla moniliformis* grows more rapidly and abundantly than the other species, and is not fastidious in its food requirements but does not produce proteolytic enzymes (gelatin; casein-agar tests).

Vitreoscilla paludosa n.sp. The species was detected in November 1947 in material from the mill-pond above Flatford Mill Field Centre after it had been kept in the laboratory for a month. It had previously contained Diatoms and Myxophyceae, *Trachelomonas*, *Phacus*, *Pandorina*. Plates were inoculated in two ways: at separate points, and by streaking. After 1 day, threads and tongues composed of many trichomes had grown out on 0·1 % beef extract and on 0·2 %

yeast extract agar. On one of the streaked plates isolated colonies had developed, from which slopes were inoculated with a capillary pipette, and thus pure cultures were obtained.

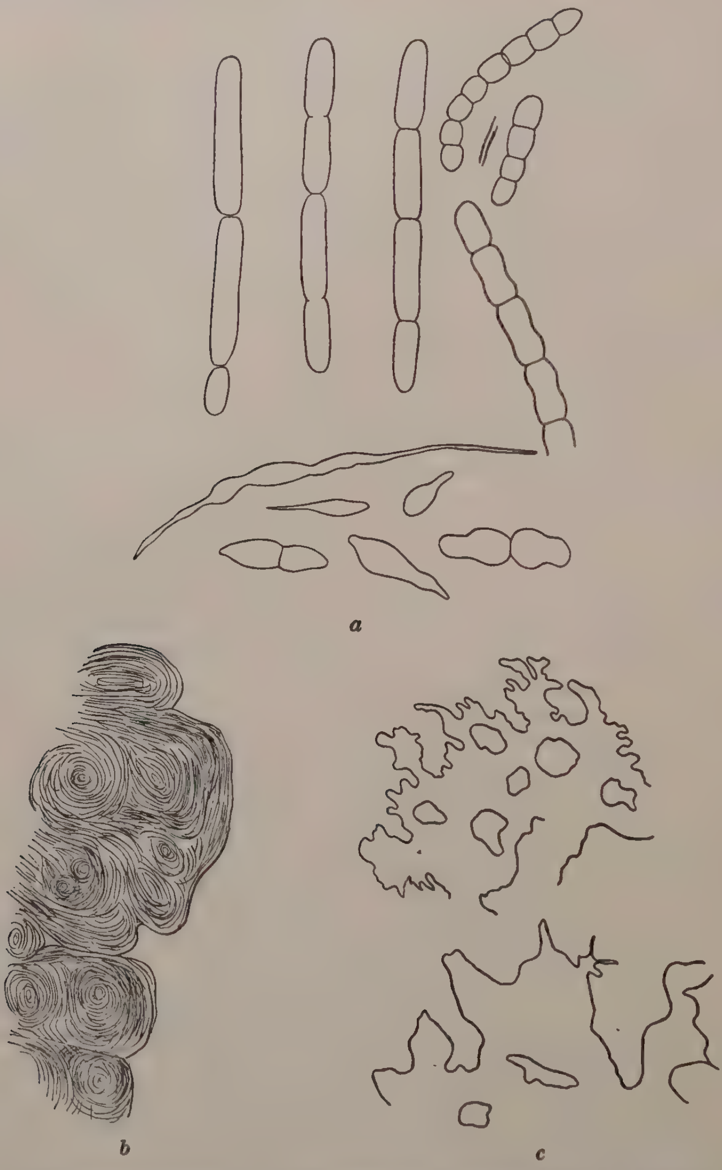


Fig. 5. *Vitreoscilla moniliformis*. *a*. Trichomes and involution forms; × 1500. *b*. Colony on yeast extract agar, 10 days old; × 100. *c*. Outlines of colonies on 0·2 % yeast extract and 0·1 % beef extract agar respectively; × 150.

The appearance of the growths in liquid media and on agar recalls that of *Beggiatoa* which can be grown and isolated in the same way (Cataldi, 1940). On thinly inoculated agar young aggregations develop which are composed of parallel trichomes (Fig. 6*a*), and grow to form elongate, slender, pointed, often Y-shaped colonies (Fig. 6*b*). Broadening occurs by the trichomes snapping on reaching a certain length, and the fragments slipping along one another; branching of colonies originates by one of the trichomes being thrust aside by the resistance of friction, and starting a new bundle. Older colonies have an inner concentric structure reminiscent of brain convolutions. At the edges they show flame-like processes and grow out into long curved and anastomosing tongues, from which again single trichomes emerge, commencing further systems of growth (Fig. 6*c*). The colonies are different from those of *Beggiatoa* in not producing curls and spirals at their fringes. In liquid media the trichomes may cover the glass surface with a film, or form cotton-like floccules, and eventually produce a sediment on the bottom.

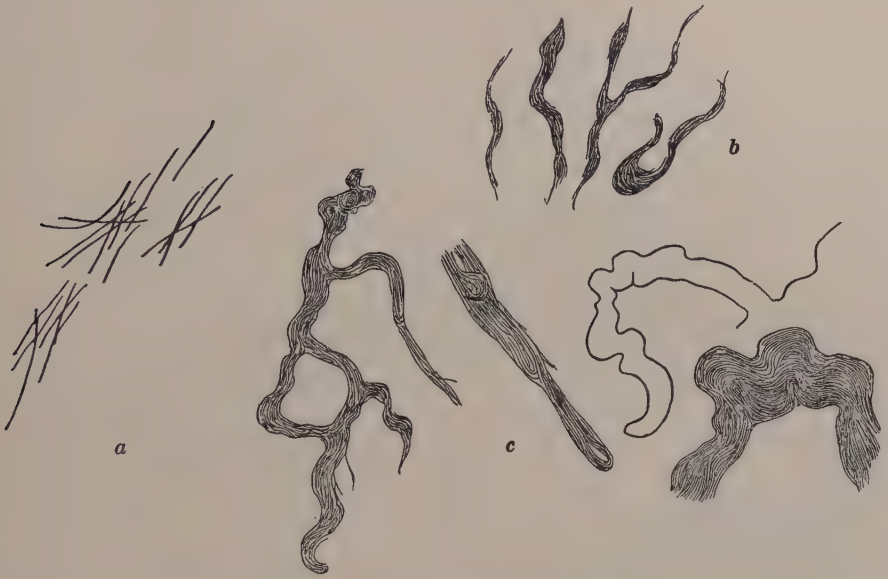


Fig. 6. *Vitreoscilla paludosa*. *a*. Bundles of straight trichomes; ×150. *b*. Younger, and, *c*, older colonies; ×150; details ×300.

The trichomes are 1·8–2 μ. wide and may become very long, 300 μ. and more in length. They subdivide into cylindrical, often slightly curved cellular sections, 30–70 μ. in length, separated by deep constrictions (Fig. 7*a*). At these places they could be observed to break during movement after bending at various angles. In one of the cultures grown in 0·1 % beef extract medium definite branching was observed (Fig. 7*b*). Old cells contain spherical granules or droplets. The cell structure is the same as that of *Vitreoscilla moniliformis*. In an attempt to stain them negatively, with nigrosin + formalin, the cells collapsed and flattened in drying, often at right angles in neighbouring cells. If, however, they were first dried and then covered with nigrosin their appearance was better preserved, outlines and constrictions becoming clearly visible.

The movement of *V. paludosa* grown in liquid media may be very lively. In yeast extract 0·2 % + Bactotryptone 0·2 % + soil extract the trichomes were extremely active in hanging drops, after attaching themselves to the surface (Fig. 8). Like all the other species under a cover-slip they ceased moving after a short time. *V. paludosa* is not exacting in its food requirements, although it does not produce proteolytic enzymes. On bacteriological agar it grows so luxuriantly that it can scarcely be distinguished from *Escherichia coli*, while on 0·1 % beef extract agar its growth is poorer but conspicuous by its filamentous structure. It also grows well and rapidly in liquid media.

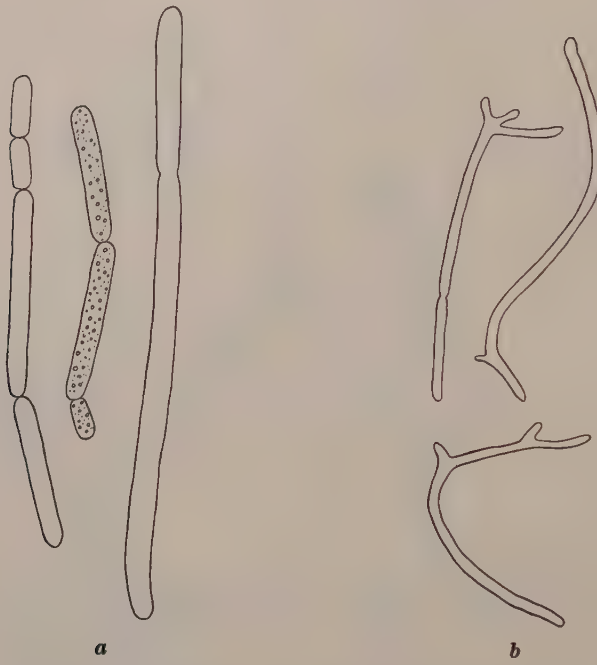


Fig. 7. *Vitreoscilla paludosa*. *a*. Trichomes, one of them showing granules; ×1500. *b*. Branching as an anomaly; ×600.

The species does not seem to be rare. Similar trichomes were repeatedly seen among the debris of raw cultures.

Vitreoscilla stercoraria n.sp. This species was isolated from dung; hence the name. In trying to isolate *Caryophanon* from cow droppings (Pringsheim & Robinow, 1947) in May 1946, particles of dung were seeded on Difco nutrient agar with 0·2 % yeast extract. After two days streptococcus-like chains had spread out upon the surface of the agar; these soon formed beautiful curls and vortices. It was easy to transfer pure material to new plates with a capillary pipette and from these to agar slopes in order to obtain bacteria-free cultures.

The trichomes of this species are about 1·2–1·5 μ. across and of varying length up to 100 μ. Their appearance is inconstant, they may be composed of rods of considerable length, 12 μ. and more, or of cells which are not much longer than they are wide (Fig. 9 *a*). The terminal cells show no more peculiarity than in the other species of the genus; the cell structure is also the same. If above a certain length the trichomes rarely remain straight but bend irregularly forming aggregates like curled hair (Fig. 9 *b*, *c*). The movement is hesitant and slow.

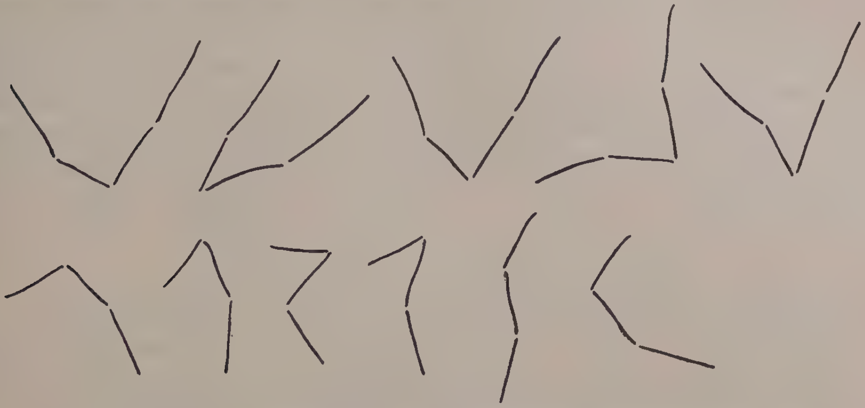


Fig. 8. *Vitreoscilla paludosa*. Changing appearances of two trichomes; × 135.

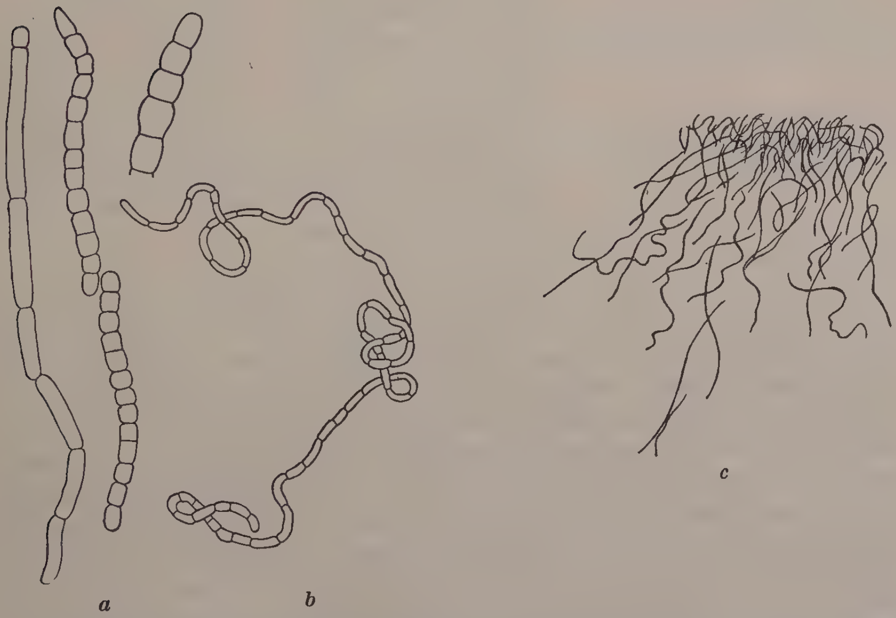


Fig. 9. *Vitreoscilla stercoraria*. *a*. Trichomes of various appearances; × 1250. *b*. Long, irregularly bent trichome, characteristic of the species; × 700. *c*. Edge of a floccule, giving the same wavy and looped appearance; × 100.

V. stercoraria grows more abundantly than *V. catenula* which it rather resembles, although it is somewhat wider and more liable to produce irregular cells. It also forms on agar denser colonies connected by a fine network, a very characteristic feature. The formation of almost regular spirals is again similar to that of *V. catenula*, but the colonies become thicker on a rich medium and, after some time, grow yellowish and translucent, owing to autolysis of the cells. The filamentous appearance is, as usual, more noticeable on low concentrations of nutrients, e.g. 0·05 % yeast extract and 0·1 % beef extract agar. On higher concentrations of yeast extract, up to 0·5 %, particularly when supplemented with peptone and on bacterial nutrient agar, the growth becomes very abundant. In liquid media the growth is at first filamentous and loose but later becomes more flocculent. The variability of appearance is considerable (Fig. 9).

Species of Vitreoscilla *isolated in pure culture*

Name of species	Width (μ.)	Morphological features	Culture appearance
V. beggiatoides	1·2	Straight trichomes; cells longer than wide	Curls and tongues
V. catenula	1·2	Moniliform; fragments readily	Curls and spirals
V. filiformis	1·2	Long, tough trichomes; cells longer than wide	Circular twirls and long loops
V. moniliformis	2·3	Straight trichomes with short or elongate cylindrical cells	Diffuse growth or locks and tongues
V. paludosa	1·8–2	Straight trichomes with long cells	Colonies with concentric structure, no curls
V. stercoraria	1·2–1·5	Trichomes often irregularly bent, moniliform or composed of sausage-shaped cells	Colonies spiral, connected by fine threads

Species not grown in pure culture

The following organisms could not be grown in pure culture and are included in the genus only with reservations.

Vitreoscilla major, nom.prov. A test-tube containing starch, soil and water was inoculated in September 1940 with half-dried mud from a ditch at Cherry Hinton, Cambridge, and kept in the dark at 25°. Two months later, following a profuse development of various Astasiaceae, a very peculiar growth appeared, consisting of tufts radiating from a point on the surface of the mud and composed of relatively long, colourless trichomes, 6–7μ. wide (Fig. 10*a*,*b*). The appearance recalled that of *Thiothrix* from which, however, the organism differed by its continuous active bending and twisting.

The trichomes were composed of cells, their length slightly less than their width, on the average 5μ. long, with a few refractive granules near the septa, as found in certain species of *Oscillatoria*. The terminal cells were in the form of flattened hemispheres (Fig. 10*b*). Seen from the tip, the trichomes rotated clock-wise. Particles adhering to the surface generally moved towards the middle of the trichomes where they accumulated in the same way as in

Oscillatoria and *Beggiatoa*. The organism was completely colourless and, as it had developed in the dark, it cannot have been photosynthetic. Its appearance was different from any known member of the Beggiatoaceae.

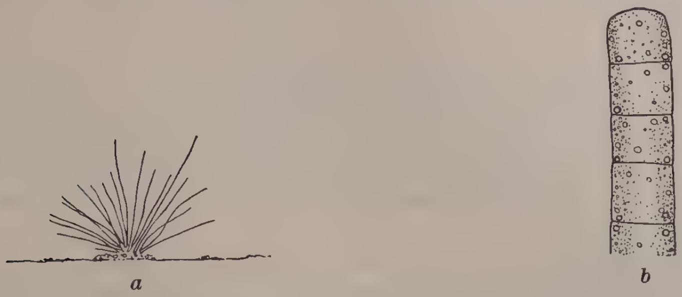
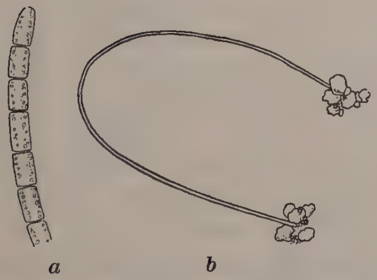


Fig. 10. *Vitreoscilla major*. *a*. Tuft; ×150. *b*. End of trichome; ×1250.



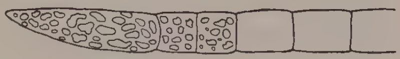


Fig. 12

Fig. 11. *Vitreoscilla stricta*. *a*. End of trichome; ×1250. *b*. Trichome caught at both ends between debris particles; ×300.

Fig. 12. *Vitreoscilla conica*. End of trichome; ×1250.

Vitreoscilla stricta nom.prov. This organism, which was found at Wray Castle and at Flatford Mill, has a diameter of 1·6–1·8μ. and forms relatively short trichomes of up to 30μ. which bend slowly and only to a small extent. True locomotion has not been observed (Fig. 11*a*). The sluggishness of the trichomes seems to be correlated with their relative stiffness. They generally adhere to the detritus with one end while the other oscillates slowly. When both ends of a trichome were caught between particles it formed a bow and gradually freed itself by stretching before swinging in the ordinary way (Fig. 11*b*). The cells are clearly seen. They are on the average one and a half times as long as they are wide. The terminal cell is not very different from the rest but is rounded at the tip.

Vitreoscilla conica nom.prov. was found in mud from South Africa, very obligingly sent to our laboratory by Dr M. A. Pocock for the growth of *Volvox* and *Hydrodictyon*. It had been collected from a pond (Du Klip Vlei, Cape Flates, Cape Province) which had dried out during the dry season and was again moistened in the laboratory.

The trichomes of *Vitreoscilla conica* are about 4μ. wide and never very long, generally 20–50μ. in length (Fig. 12). The cells are slightly barrel-shaped and filled with refractive pseudo-vacuoles. The end cell is often longer than the rest and attenuated towards the tip, hence the suggested specific name. The gliding movement was not active but, since its speed generally depends on temperature, this observation does not necessarily mean that the organism is equally sluggish in its natural habitat.

MICROSCILLA n.gen.

Since only one of the forms included in this genus could be grown in pure culture, the names should be considered as provisional. Most of the species were observed repeatedly but their identities could not be finally established owing to their relative lack of recognizable characteristics. The relation to Soriano's *Flexibacter* remains doubtful.

Microscilla marina n.sp. This organism appeared in November 1947 on plates of sea-water Schreiber agar inoculated with fragments of *Schizonema* (Diatom) filaments from Brighton. Colourless trichomes grew out upon the exposed areas of the agar surface and eventually could be freed from other organisms including bacteria. The organisms multiplied better when 0·05 % Difco yeast extract or beef extract or various peptones were added, but not in higher concentrations than 0·2 %. Glucose did not have an enhancing effect; acetate supported slightly richer growth. Without sea-water there was no development at all. Sea water diluted 50 % was, as in several other instances, slightly better than at full concentration. Sea water at double and one-quarter concentration permitted slight growth. Artificial sea water, although only approximately the natural composition, was quite as good as natural sea water. These observations, and those on *Beggiatoa*, on *Porphyridium*, on Myxophyceae and on various other algae and flagellates are not in accordance with van Niel's (1931, p. 31) view that there is no ecological-physiological difference between fresh-water and marine micro-organisms. This view had already been expressed by Baas-Becking (1925) and was repeated by ZoBell (1946).

Growth on agar slopes is not very conspicuous. On plain sea-water agar with dilute Benecke solution it may be almost invisible to the naked eye. The richest growth was obtained on the following medium: sodium acetate 0·1 %, Difco yeast-extract 0·1 %, Bactotryptone 0·1 %, soil-extract 5 %, artificial sea water half concentrated, agar 1 %, neutralized with calcium carbonate. After 4 days a thick layer, peach to orange in colour, was formed. In other media this was only seen at the bottom of slopes where the organism had accumulated. The rich orange growth disappeared during the next week by autolysis of the cells, while smaller growths kept their filamentous appearance for several weeks.

The appearance of the organism could be observed more in detail when grown on agar plates (Fig. 13*a–c*). With the naked eye only a greyish veil can be seen which, under the microscope, is transformed into a delicate network of widely separated trichomes forming loops and meshes. At the edge of the area of growth there appear fringes and tongues composed of filaments, and single trichomes, occasionally wound together spiral-fashion. Very characteristic is the formation of denser aggregations in the otherwise very loose network (Fig. 13*d*).

The individual trichomes are 0·5–0·6 μ. wide, up to 100 μ. long and apparently without segmentation (Fig. 14*a*). The active gliding movement is not associated with rotation but with much bending and waving. Only when gliding along a glass surface may the trichomes become almost straight with slight, serpentine undulations. The movement is so quick, particularly in young material from

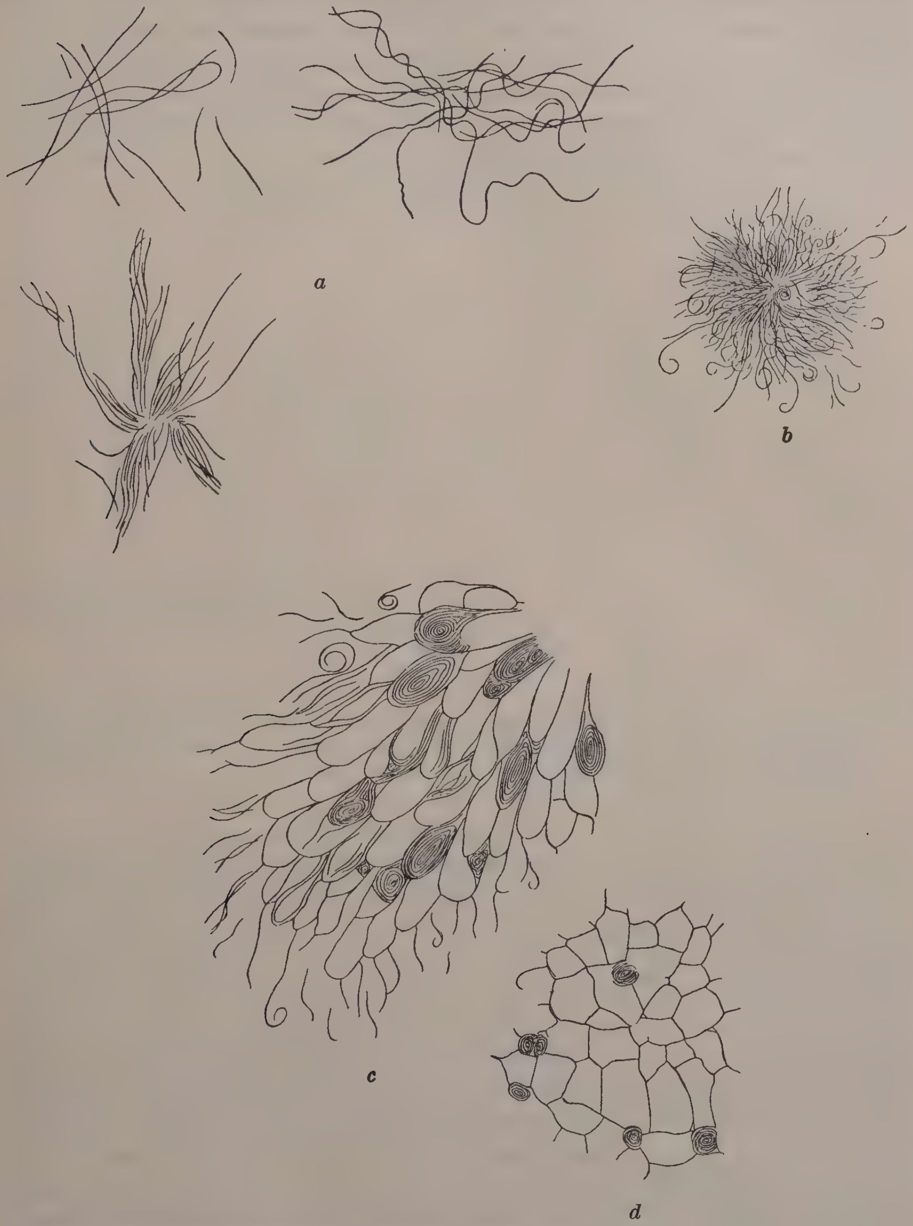


Fig. 13. *Microscilla marina*. *a*. Young colonies on agar, ×250. *b*. Older colony, ×150. *c*. Edge of old colony, yeast extract sea-water agar, ×75. *d*. Sparse growth on sea-water agar with dilute Benecke solution without addition of organic substances, ×150.

liquid media, that it was only just possible to sketch the ever-changing shape of active trichomes. In the condensation fluid of agar slopes and in liquid media floating bundles of almost parallel trichomes are formed which continually glide along one another turning and twisting in various directions (Fig. 14*b*). When accidentally bent, they recovered their shape with great rapidity, but no free swinging ends were ever observed. The organism is delicate and had to be subcultured every week; it was lost during the hot summer of 1949. Since no effort was made to obtain the same or other marine members of the group it remains to be seen whether these can regularly be found.

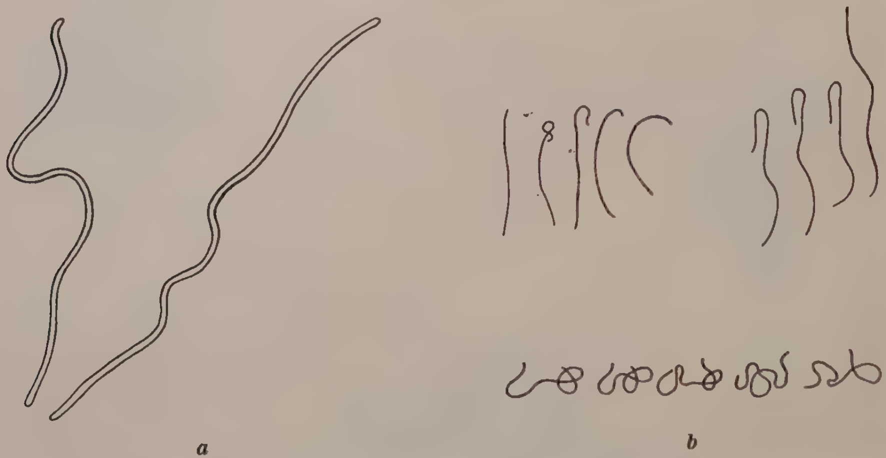


Fig. 14. *Microscilla marina*. *a*. Trichomes; × 1250. *b*. Three trichomes, consecutive stages of movement, quick sketches of changes in shape, liquid medium: proteose-peptone 0·1 % + beef-extract 0·1 % + soil-extract + sea water; × 250.

Microscilla agilis nom.prov. This organism, which was at first mistaken for a spirochaete, was found in a ditch on Coldham Common near Cambridge in August 1941. The specimen contained, between decaying plant residues, a great variety of flagellates, *Cladophora* and *Chlorohydra*. The colourless trichomes of this *Microscilla* were 12–70μ. long and 0·6–0·8μ. wide, i.e. slightly wider than those of the previous species. No segmentation could be seen either in unstained preparations or those stained by neutral red, either by direct or dark-ground illumination. The coils of spirochaetes in the same field could clearly be seen, although these were narrower than the trichomes of *M. agilis*. The motility and flexibility of the organism were extraordinary and were reminiscent of *Spirochaeta plicabilis* (Fig. 15*a*). Moving on the cover-slip the trichomes were almost straight, but sometimes produced regular undulations. They glided along particles which they touched, continually changing their shape. On reaching the end the trichomes reversed the direction of their movement (Fig. 15*b*). The trichomes seemed always to be attached along their entire length; free, funnel-wise swinging ends were not seen. Rotation seems to be lacking. Except for its fresh-water habitat, the species is very similar to *Microscilla marina*.

Microscilla flagellum nom.prov. This form is similar to the two just described but even more active and slender, attaining a width of barely 0·4μ. Its long trichomes are common between pigmented filamentous Myxophyceae and can almost regularly be found in their floating masses on quiet waters, often with wider, more sluggish, perhaps not always completely pigment-free and more scattered trichomes. *M. flagellum* is also found between plant debris.

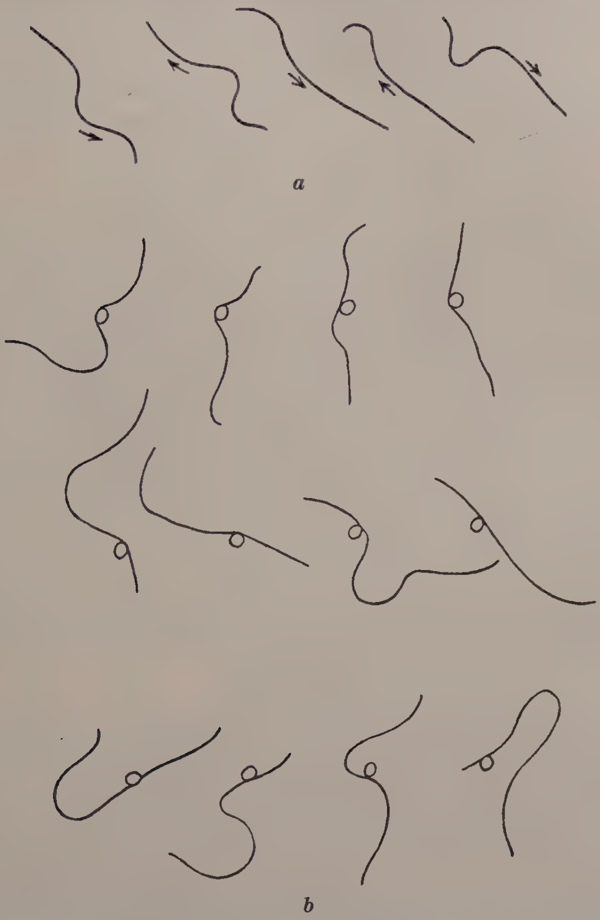


Fig. 15. *Microscilla agilis*. *a*. Sketches of quick changes in shape and in direction (indicated by arrow), exhibited by trichomes attached to cover-slip; × 500. *b*. Similar; trichome glides along particle to which it remains attached in spite of translocation; × 500.

In its usual habitat it is found in great numbers, gliding, and twisting among the much larger trichomes of *Oscillatoria*, *Phormidium* and *Anabaena* sometimes winding in a spiral around them. The extremely active movements of trichomes fixed at one end recall to some extent those of flagella of euglenoid flagellates trapped between particles of debris, hence the specific name. When gliding the trichomes continually change their shape and direction. They may lash about like a whip or turn back upon themselves to form hooks and loops.

BACTOSCILLA nom.prov.

Only one species, *Bactoscilla mobilis* nom.prov. has so far been found, and this only a few times and not in great numbers. It was first observed in scum composed mainly of Myxophyceae from the outlet of the 'Clay Pond' near the Freshwater Biological Association laboratory at Wray Castle, Ambleside, later in the surface film of water over black mud from Flatford Mill Field Centre, and a third time in mixed material from a duck-pond near Colchester.

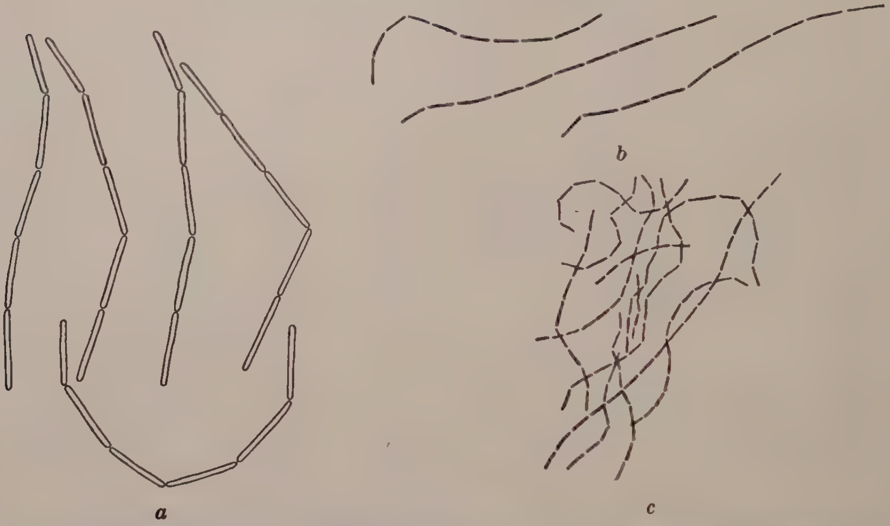


Fig. 16. *Bactoscilla mobilis*. *a*. Trichome of five rods, stages in quick change of position, and trichome of six rods, bending in joints; × 1250. *b*. Trichome of ten rods, stages in quick change; × 200. *c*. Bundle of Trichomes; × 500.

The cells of this organism are slender and rod-shaped, $0{\cdot}4\,\mu$. wide, forming chains with gaps between the component rods (Fig. 16*a*). By its gliding movement the species appears to be related to the other Vitreoscillaceae, but owing to its minuteness its details could not be resolved even with a × 120 Zeiss apochromatic oil immersion objective, × 8 compensating eyepiece and optimum illumination, or with dark-ground illumination. The gliding movement of the trichomes is similar to that of other Vitreoscillaceae. Only when they are fastened with one end, and the other one bends and twists, does the peculiarity of the movement reveal itself, its most unusual feature being the rigidity of the individual rods, while bending takes place in the joints, the exact nature of which is not clear (Fig. 16*b*, *c*). These hinge-like movements remind one of the joints of an arm, but the whole trichome curves in various directions at short intervals.

One may wonder whether other filamentous gliding organisms behave fundamentally in the same way without this being observable because the cells are too short for their rigidity to be perceived. In *Vitreoscilla moniliformis*, the largest of the genus, trichomes composed of a few cells showed something of the kind, i.e. considerable bending of the whole chain without alteration in the shape of the cells. The generally accepted explanation of myxophycean locomotion by contraction and expansion of the cells cannot therefore be applied to the Vitreoscillaceae. In the Myxobacteria, on the other hand, the movement and appearance of which is so similar (Pringsheim, 1949, p. 86), a marked flexibility of the cells is readily observed. The Wray Castle form seems to have been larger though otherwise very similar. The material was scarce, and my notes do not suffice to establish the relationship. It was also not possible to decide whether the bending was passive like that of the other filamentous gliding organisms (cf. p. 133) or active and concentrated in the joints as the appearance suggested.

FLEXOSCILLA nom.prov.

Of this genus only one species, *F. mucronata* n.sp., has been observed. It was detected in January 1942 in a culture of decaying algae from Coldham Common, Cambridge. Four months after the material had been brought in and small quantities of sodium sulphide had repeatedly been added, a large number of the organisms had grown in the bottom deposit and on the glass near the surface of the water. It glides rapidly and is a flexible, possibly unicellular rod, about 1 μ. wide and 12–15 μ. long. Its external membrane is less distinct than that of *Spirillum* present in the same preparation and is scarcely illuminated by dark ground illumination. Both ends are tapering but are not acute. While moving the rods are usually almost straight (Fig. 17).

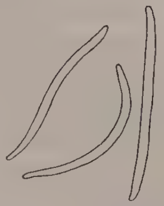


Fig. 17. *Flexoscilla mucronata.* Three specimens of various shapes which may all occur in the same individual by bending.

DISCUSSION

The search for colourless gliding organisms has led to the conclusion that there are many more species than have so far been identified. This is borne out first by the observation of these forms in various habitats; often only a small number of trichomes could be found, but these were sufficient to show that they were taxonomically distinct. Secondly, this view is supported by the fact that all the strains grown in pure culture represent different species so that further isolations should furnish a greater number of different species.

The Vitreoscillaceae will therefore probably prove to be as large a group as the Myxobacteria of which only a very restricted number has been studied. The Myxobacteria comprise members which decompose cellulose and chitin or are parasitic, but the majority live either on bacteria or on the products of bacterial metabolism. By contrast, none of the known Vitreoscillaceae seems to have special biochemical properties, but it would not be safe to say that this negative statement is final.

There is a decided similarity between the Vitreoscillaceae and the Myxobacteria which may indicate a real affinity. The two groups have the following characteristics in common: (1) lack of true cell walls composed of a substance chemically different from the cytoplasm, from which it separates in hypertonic solutions; (2) low refractivity due to high water-content and possibly to the absence of a rigid cell wall; (3) gliding movement, without observable cause and based on at least partial adherence to solid bodies or the water-air interface; (4) the more-or-less pronounced trichome formation.

There are also distinct morphological differences between the Vitreoscillaceae and the Myxobacteria, indicating a higher organization in the latter: (1) the so-called microcysts which perhaps all species are able to produce; (2) the fruiting bodies of the higher Myxobacteria. Most of the Vitreoscillaceae are also more pronouncedly filamentous, some of them even forming threads of great length while the majority of the Myxobacteria produce only elongate rods, and the trichomes formed by a few of them are only short.

In respect of filament formation some of the Vitreoscillaceae resemble the Beggiatoaceae which also glide in a similar way. *Beggiatoa alba*, the best-known species, is different from most species of the Vitreoscillaceae, though not from *Vitreoscilla filiformis*, in the unrestricted length of the smooth, cylindrical trichomes while most species of *Vitreoscilla* have more or less rounded cells and shorter trichomes. This phenomenon has, however, also been observed in *Beggiatoa mirabilis* (cf. Benecke, 1912, p. 205), so that there is no fundamental difference in this respect. The similarity between trichomes of *Vitreoscilla* and those of *Beggiatoa* when sulphur-free, may be so great that one might easily be mistaken for the other. In fact, *Beggiatoa alba* can be isolated and grown in pure culture in much the same way as *Vitreoscilla* (Cataldi, 1940; personal observations).

The taxonomic position of the Vitreoscillaceae seems to be intermediate between the Myxobacteria and the Beggiatoaceae, which are most probably apochlorotic descendants of Myxophyceae (Pringsheim, 1949, p. 63). Before we know more of their cell organization the true relationship between the three groups cannot be established. Cytological investigations, in which Mrs Klieneberger-Nobel (Lister Institute, London) and Dr C. F. Robinow (then at the Strangeways Laboratory, Cambridge) very kindly assisted me, proved to be as difficult as in the case of Myxophyceae. One of the reasons is the inconstancy of the morphological features of the Vitreoscillaceae. It is nevertheless certain that Feulgen-positive nuclear structures are present, two in each cell but sometimes more in inflated forms (Pl. 3, figs. 5–7).

A constant feature of Vitreoscillaceae as of Beggiatoaceae and Myxobacteria is the mucilage production which appears to be connected with the gliding movement, since in all organisms exhibiting locomotion of this kind the cells are embedded in a slimy mass. They also leave tracks which can clearly be seen on agar. Otherwise the mechanism of the movement is as mysterious as that of other gliding organisms.

The ease with which these organisms can be kept and grown makes them valuable objects of research into cell physiology and nutrition. In order to facilitate such work cultures of *Vitreoscilla*, *Beggiatoa* and Myxobacteria are kept for general use in the Culture Collection of the Botany School, Cambridge.

DIAGNOSES

VITREOSCILLACEAE Pringsheim nov.fam., colourless, filamentous, gliding micro-organisms, differing from Myxophyceae in lacking assimilatory pigments, and from Myxobacteria by a more pronounced trichome formation and the absence of microcysts. Type genus *Vitreoscilla* Pringsheim.

Vitreoscilla Pringsheim, nov.gen. Trichomes clearly subdivided into cells. Reproduction by hormogonium-like trichome fragments. The sites of fragmentation are usually indicated beforehand by contractions between the cells.

(1) *V. beggiatoides.* Trichomes 1–2μ. wide, may become very long. They are subdivided by shallow constrictions. Cells elongate, cylindrical, varying much in length. Growth on agar composed of long trichomes forming curls and projections. Type-strain isolated in June 1941 from cow dung. The same species isolated again from:

(2) *V. catenula.* Trichomes 1·2–2μ. wide, often moniliform, never very long, fragmenting in a variety of ways. Grows on agar in spirals and whorls. Type strain isolated in November 1945 from cow dung. The same species was found in an infusion of plant material from:

(3) *V. filiformis.* Trichomes 1·2μ. wide, very long and tough, cylindrical. Cells short, cylindrical. On agar circular spirals and loose loops. Isolated in February 1946 from soil, but also occurring in water.

(4) *V. moniliformis.* Trichomes 1·8–2·5μ., rarely up to 3μ. in diameter, usually about 25–40μ. long, but sometimes up to 150μ. Cells vary from spherical to rod-shaped, usually rounded so that trichomes are moniliform. On agar rich in nutrients, diffuse growth spreading in zones. When less well nourished filamentous loops and projections. Isolated from cow dung in June 1947.

(5) *V. paludosa.* Trichomes 1·8–2μ wide, often very long, up to 300μ., sometimes even more, composed of rod-shaped cells 30–70μ. in length. Colonies on agar often showing concentric structures with the edge growing out into projections and curls. Isolated in November 1947 from water with decaying algae.

(6) *V. stercoraria.* Trichomes 1·2–1·5μ. wide, sometimes involuted to 1·8μ. of varying length, up to 100μ. They are composed of cells, the length of which is from about the same as the diameter to 12μ. and more. Growth on nutrient agar luxuriant. Colonies spiral, characteristically connected by a fine network of trichomes, especially on a less rich agar medium. Isolated from cow dung in May 1946.

Microscilla nov.gen. Trichomes narrow without perceptible septation. Gliding movements active. Reproduction by division into relatively long daughter trichomes. Type species *M. marina.*

M. marina. Trichomes 0·5–0·6μ. wide, up to 100μ. long, cylindrical. Constrictions few, occurring only before division. On agar, translucent colonies composed of loops and whorls. Type strain isolated in December 1947 from marine algae on chalk in the tidal zone at Brighton, Sussex, England.

Bactoscilla nov.gen. Trichomes narrow, composed of rod-shaped sections which are relatively rigid and held together by an invisible, pliable connexion. Type species *B. mobilis.*

Flexoscilla nov. gen. Trichomes short, possibly unicellular, tapering at both ends. Type species *F. mucronata.*

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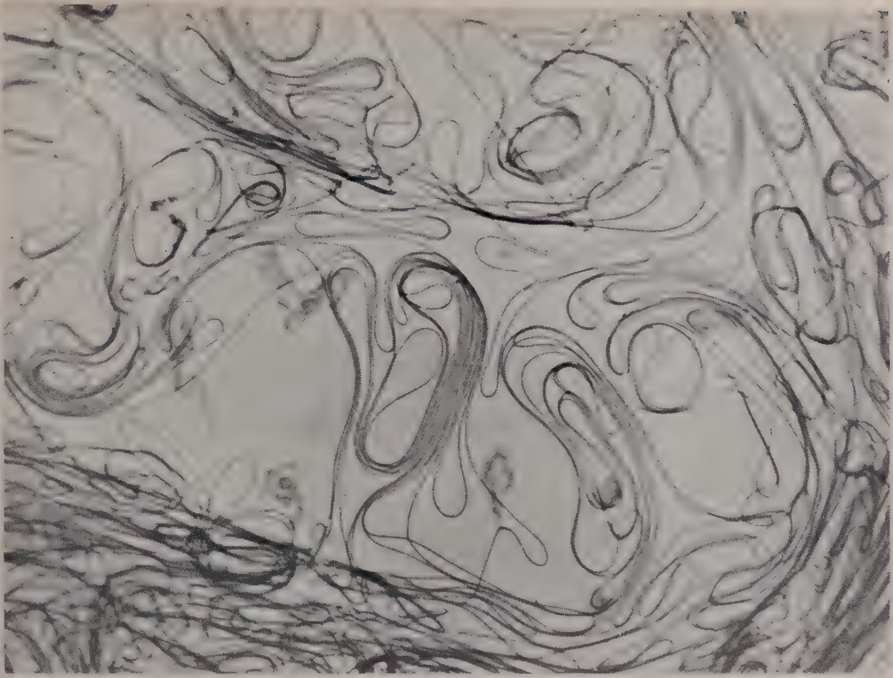

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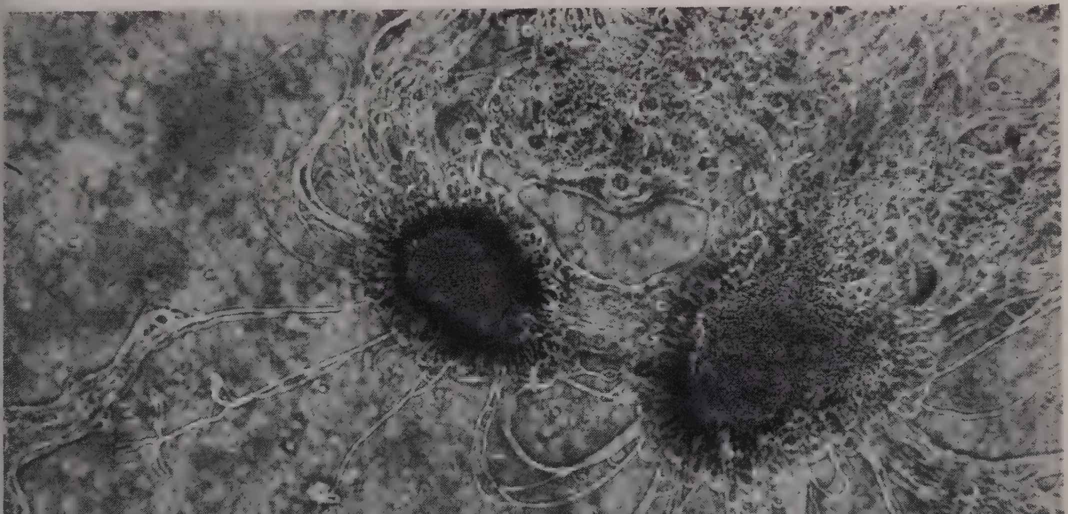
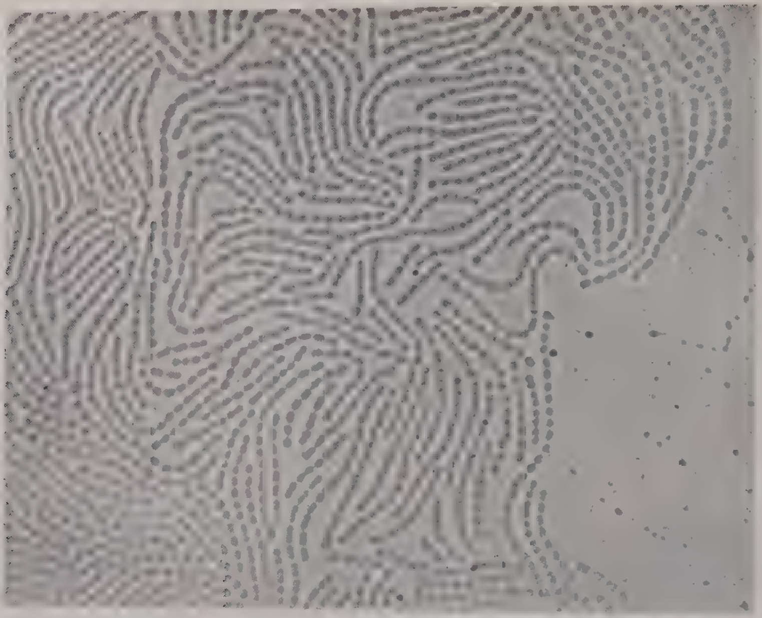


Fig. 2

E. G. PRINGSHEIM—THE VITREOSCILLACEAE. PLATE 1



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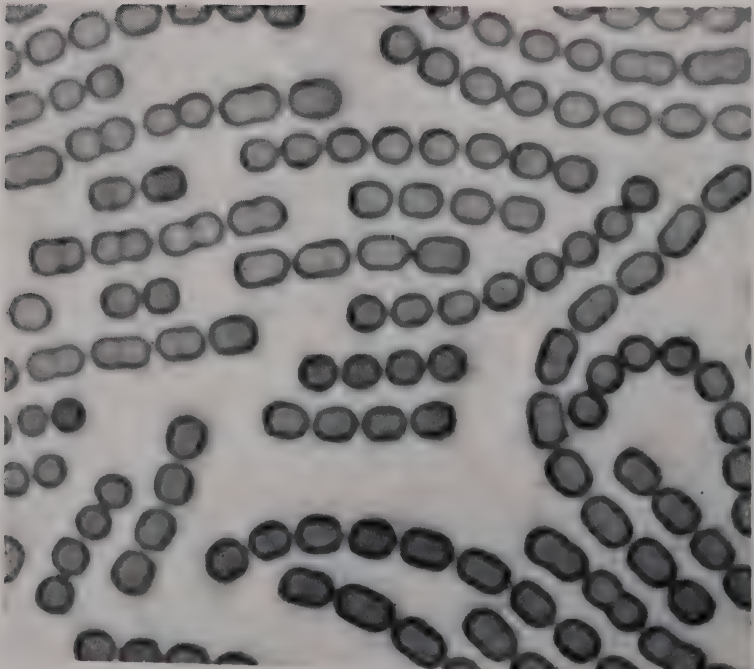

Fig. 4

E. G. Pringsheim—The Vitreoscillaceae. Plate 2

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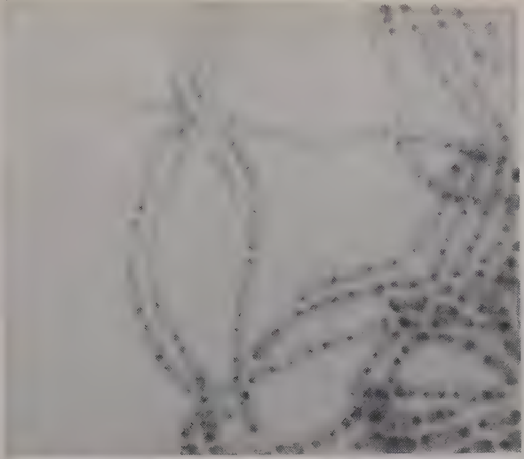

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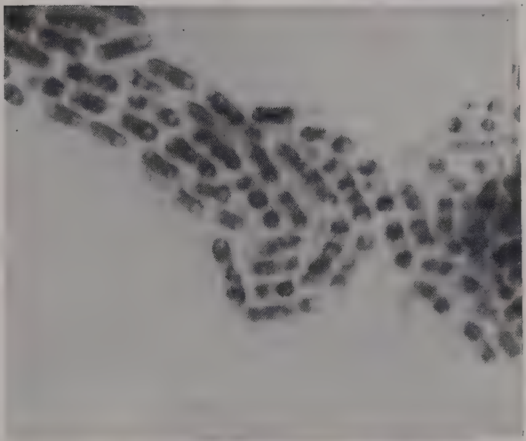

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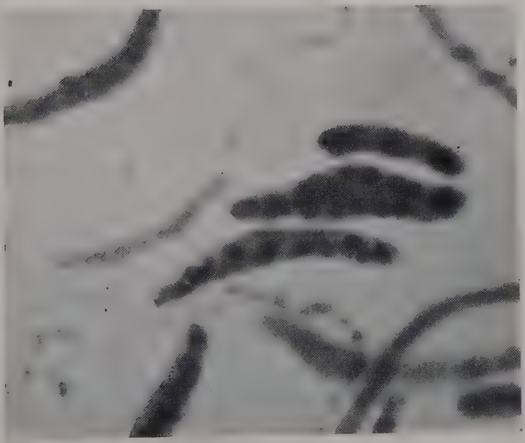

Figs. 5–7.

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EXPLANATION OF PLATES

Plate 1. *Vitreoscilla filiformis*

Fig. 1. Part of large colony on nutrient agar, Bouin Giemsa; × 420 (prep. and phot. Robinow).

Fig. 2. Trichomes growing out of soil particles on agar plate (phot. Rothamsted).

Plate 2. *Vitreoscilla moniliformis*

Fig. 3. Edge of living, 3-day-old colony; × 720 (phot. Robinow).

Fig. 4. Part of the same colony, Bouin tannic acid + crystal violet; × 3000 (prep. and phot. Robinow).

Plate 3. *Vitreoscilla stercoraria*

Fig. 5. Meat infusion agar, 18 hr., 37°, osmic acid thin Giemsa.

Fig. 6. Yeast extract agar, 24 hr. room temperature, osmic acid HCl Giemsa.

Fig. 7. Yeast extract agar, 3 days room temperature, osmic acid thin Giemsa (prep. and phot. Klieneberger-Nobel).

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The Development of Bacterial Flagella in the Germinating Microcyst

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SUMMARY: The microcyst stage of *Salmonella typhi*, *Proteus vulgaris*, *Bacterium coli* and *Pseudomonas fluorescens* is non-motile and devoid of flagella. Observations by electron- and photomicroscopy indicate that the flagella grow outwards through the cell wall of the germinating microcyst. They commence to develop after about 2 hr. incubation. Alike in bacteria with peritrichous and polar flagella a single, polar or sub-polar flagellum develops first. In the process of maturation the flagella are shed and the non-motile microcysts produced. Basal granules may occasionally be observed.

In recent years there has been some controversy about the nature of bacterial flagella, centring upon the work of Pijper (1946), which was discussed in the recent Symposium (1949) on 'the nature of the bacterial surface' held by this Society, and which need not be further reviewed here. The present study shows that bacterial flagella grow outwards through the cell wall of the developing microcyst, and that their development follows a regular pattern.

METHODS

Inocula, made from cultures of *Salmonella typhi*, *Proteus vulgaris*, *Bacterium coli* and *Pseudomonas fluorescens*, appeared microscopically to be composed mainly of microcysts (Bisset, 1950*a*, *b*), and were entirely non-motile. Various strains took from 2 or 3 days to several weeks to achieve this condition. Inocula were washed in saline and centrifuged to free them from loose flagella derived from the previous, vegetative generation. Cultures were made on nutrient agar, and from these, preparations were made at various periods after inoculation. Preparations were stained by a variety of 'flagellar impregnation' stains, of which mordanting with a solution of 7 % tannic acid and 0·5 % ferric chloride, before heating with Hortega's silver solution, was found to be most satisfactory. Preparations were also examined with the electron microscope. These were shadowed with metallic gold at an angle of 45 degrees. Lower angles gave greater apparent clarity of image, but obscured details in the margins of the bacteria. Motility was examined in hanging drops.

OBSERVATIONS

Germinating microcysts. Mature microcysts, before germination, were entirely devoid of flagella (Pl. 1, fig. 1). Motility began to develop at about 2 hr. incubation at 37° and bacteria from these cultures showed all stages of development of flagella. Some just pierced the cell wall (Pl. 1, figs. 2–5; Figs. 1, 2). In some cases the emergent flagella appeared to push the cell wall outward, forming a collar around the base of the flagellum. In *Salm. typhi*, *Pr. vulgaris* and *Bact. coli* the first to appear were polar or sub-polar, and almost always the remaining flagella developed most rapidly in the region of this pole. There is evidence that the main growth of the cell was from the opposite pole (Bisset, 1951) (Pl. 1, fig. 3, and Pl. 2, figs. 6–10). The flagella of *Ps. fluorescens* were exclusively polar or sub-polar in position, but here also one usually emerged well in advance of the remainder (Pl. 2, fig. 11, and Pl. 3, figs. 12–15). After a short time, in all these species, some flagella could be observed to be fully grown, while others were in various stages of growth (Pl. 3, figs. 16–20), and in the case of *Ps. fluorescens* they occasionally began to appear at the opposite pole also, well in advance of their normal appearance at cell division (Bisset, 1951) (Pl. 3, figs. 21, 22, and Pl. 4, fig. 23). Mature bacteria, with their full quota of flagella, were common in cultures aged 4 hr. and more. In *Salm. typhi* they were regularly ten in number, distributed evenly along the sides of the bacterium, rather towards one pole; those of *Ps. fluorescens* were four or five in number, in a tuft at one pole; those of *Pr. vulgaris* were more variable, and often were exceedingly numerous (Pl. 4, figs. 24–26).

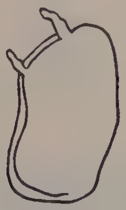


Fig. 1

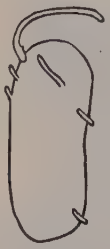


Fig. 2

Fig. 1. Tracing of germinating microcyst of *Proteus*.

Fig. 2. Tracing of germinating microcyst of *Sal. typhi*.

Older cultures. Cultures more than 2 or 3 days old, the exact age depending upon the strain, began to contain bacteria in process of maturation into microcysts. During this process the flagella were shed, and in preparations from such cultures, large numbers of broken, cast-off flagella could be seen (Pl. 4, fig. 27). These could be removed entirely by washing and centrifuging.

Basal granules. Occasional electron micrographs of germinating microcysts showed granular structures at the base of the flagella (Pl. 1, fig. 5), and one partially disrupted cell of *Ps. fluorescens* showed these basal granules or blepharoplasts very clearly (Pl. 4, fig. 28).

DISCUSSION

The observation that the resting cells or microcysts of motile bacteria are non-motile and devoid of flagella provides a starting-point for the examination of the development of the flagella. They begin to appear after about 2 hr. incubation of mature microcysts when these are subcultured upon fresh medium. The pattern of development is surprisingly regular (Fig. 3). Almost always the polar flagella develop before the remainder. Partly-grown flagella often appear, in electron micrographs, to have a collar around the base. This may be an artefact, but as it is common in half-grown flagella, and rare when they are fully developed, it may be due to mechanical pushing outwards of the cell wall by the growing flagellum, or may even be a structure concerned with the secretion of the flagellum. When the vegetative bacterium matures into a microcyst the flagella are cast off, and may break up into short pieces. The existence of the bacterial blepharoplast, described by Houwink & van Iterson (1950), is confirmed.

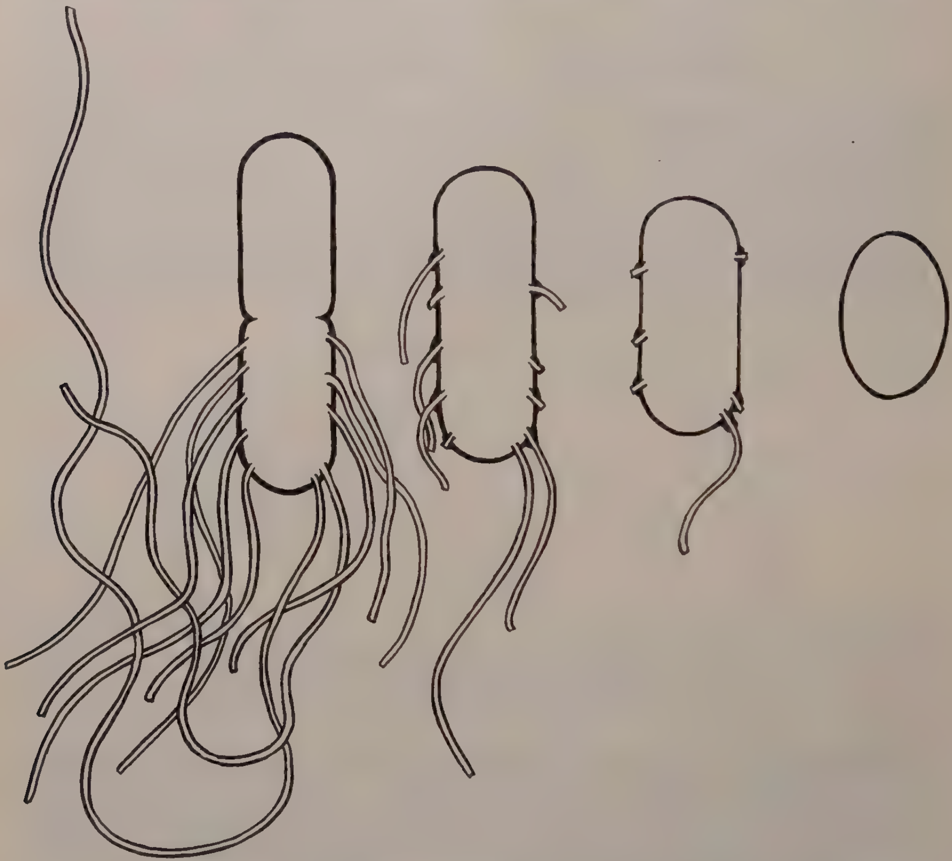
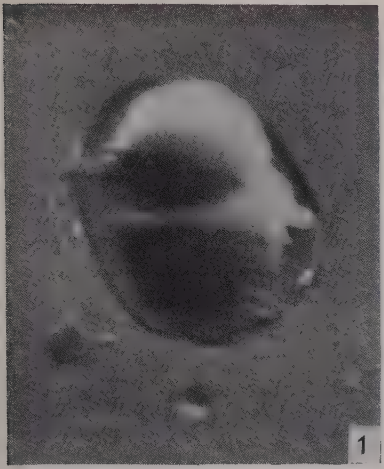


Fig. 3. Development of the flagella upon the germinating microcyst of *Sal. typhi.*

All these points indicate that the flagella are organs, produced within the cell wall and growing outwards through it, and that their arrangement and development are regular and characteristic. The observations of Pijper (1946) that the flagella may become twisted into a polar whip, visible by dark-ground illumination, are perfectly compatible with these conclusions, but Pijper's hypothesis that the flagella are threads of mucus, drawn out from the slime layer of the bacteria, is utterly at variance with the evidence presented in this paper.



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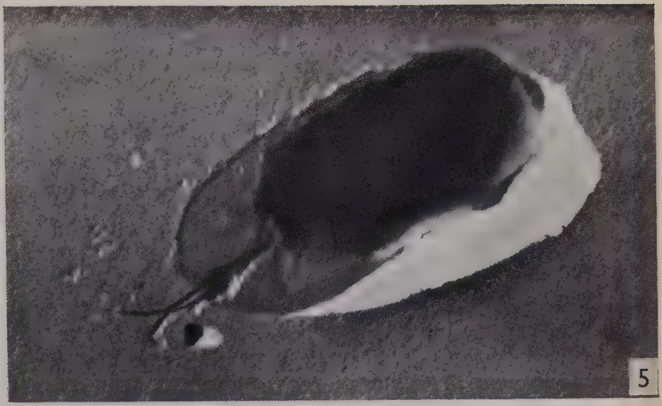

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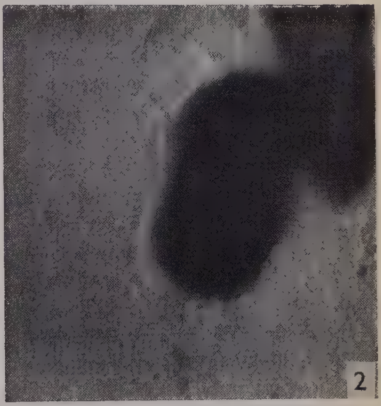

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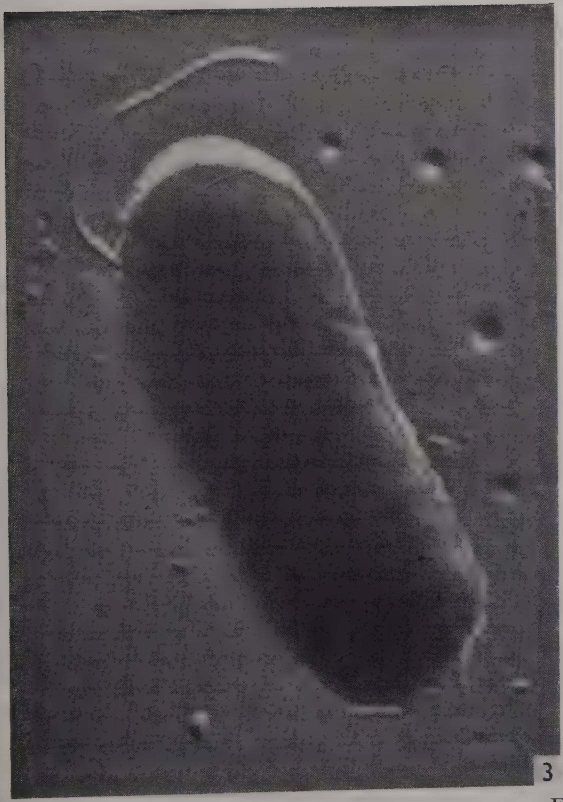

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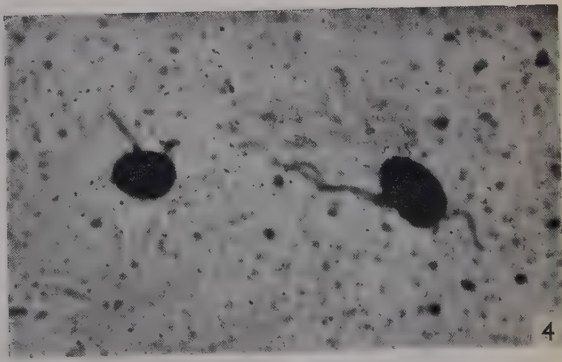

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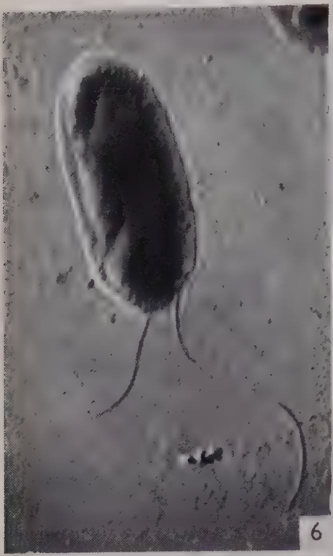
Figs. 1–5

K. A. BISSET AND C. M. F. HALE—FLAGELLA OF GERMINATING MICROCYSTS. PLATE 1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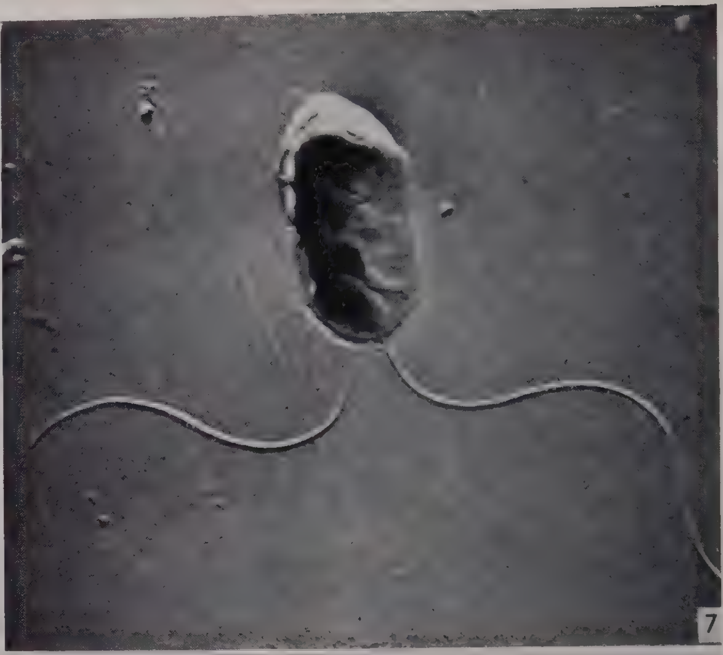
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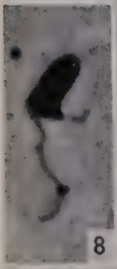
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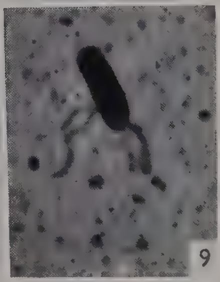
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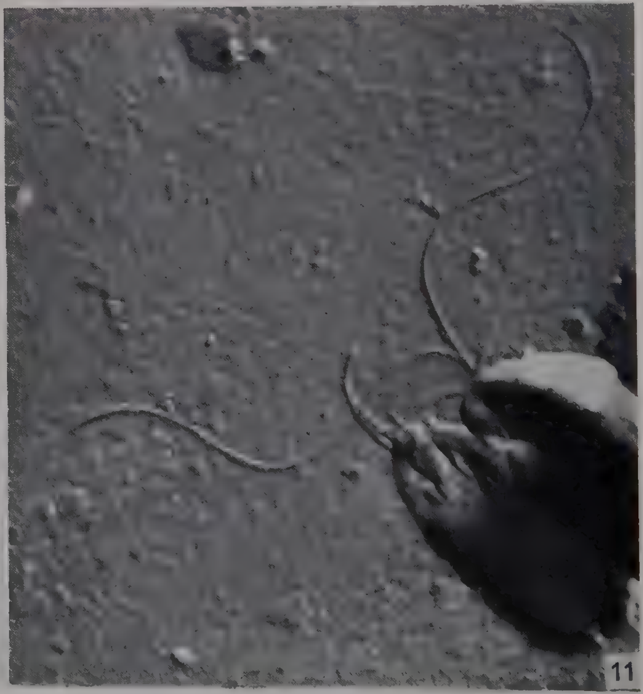
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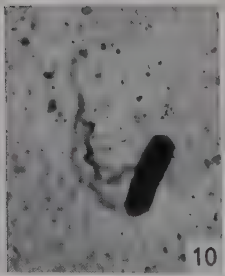
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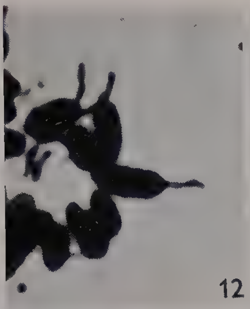
Figs. 6–11

K. A. BISSET AND C. M. F. HALE—FLAGELLA OF GERMINATING MICROCYSTS. PLATE 2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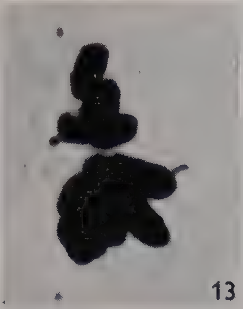
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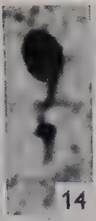
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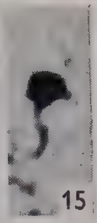
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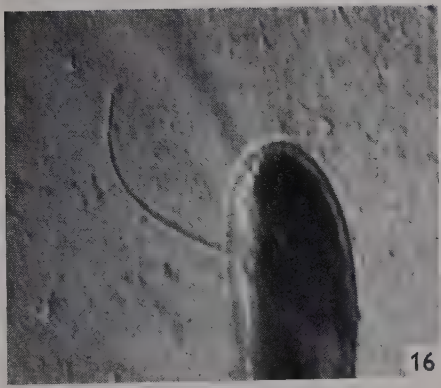
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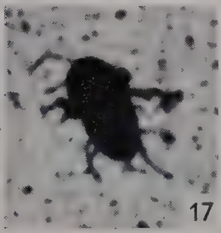
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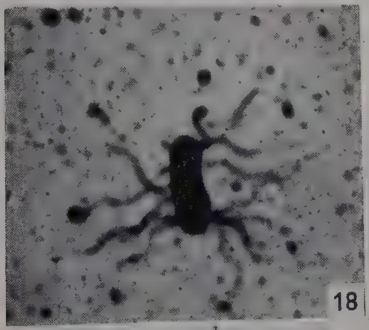
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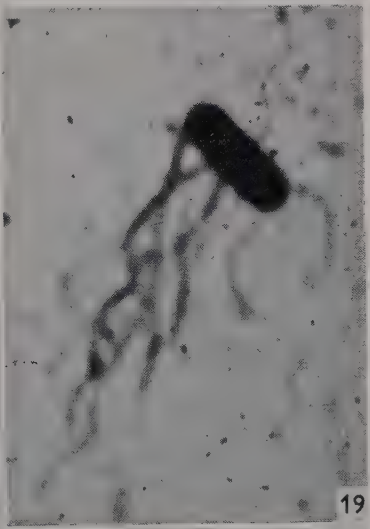
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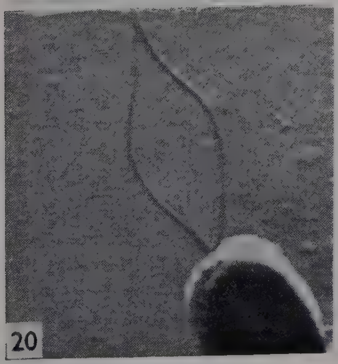
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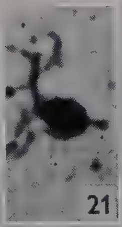
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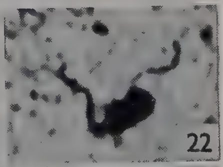
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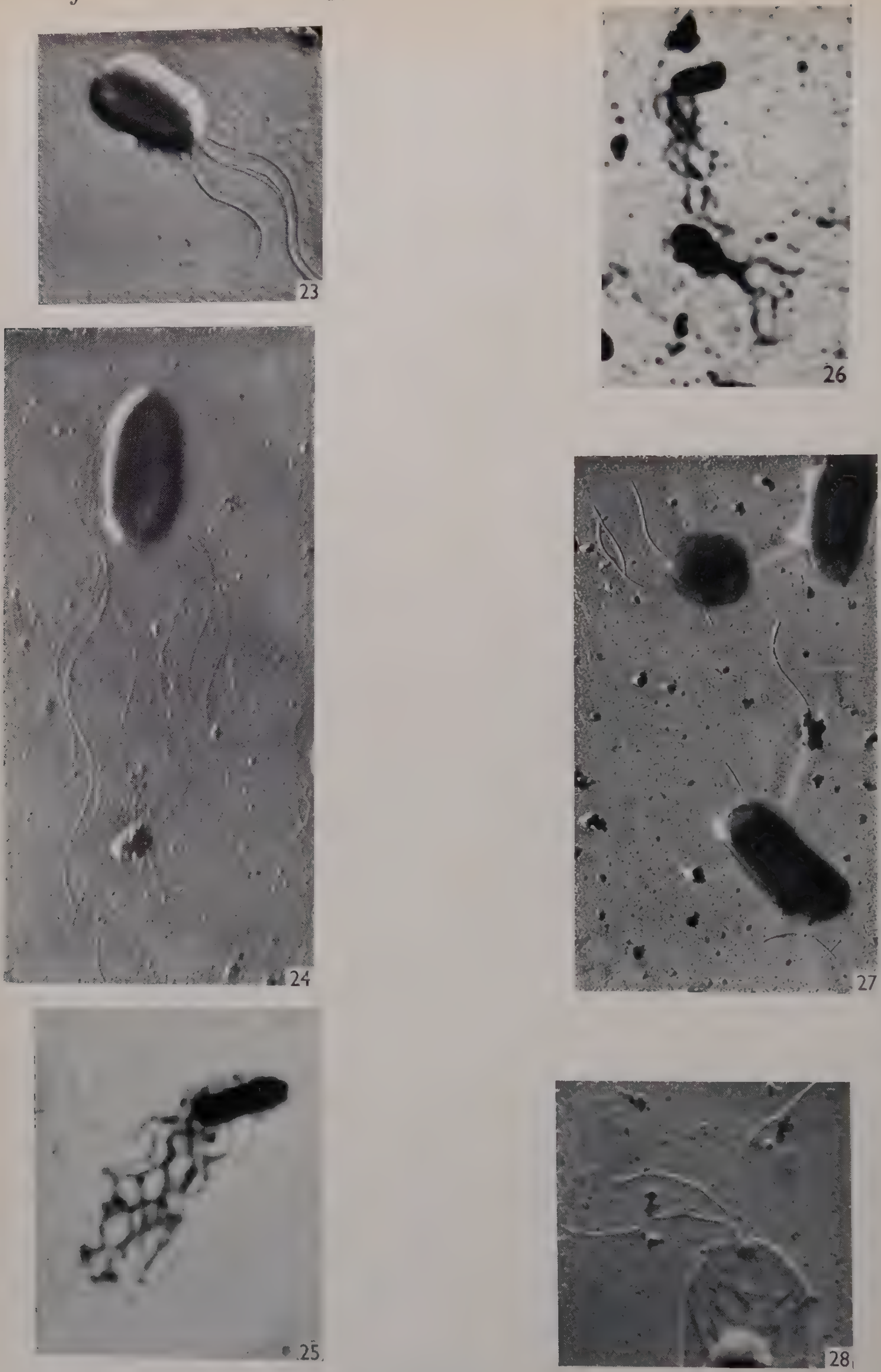
Figs. 12–22

K. A. Bisset and C. M. F. Hale—Flagella of germinating microcysts. Plate 3



Figs. 23–28

K. A. BISSET AND C. M. F. HALE—FLAGELLA OF GERMINATING MICROCYSTS. PLATE 4

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EXPLANATION OF PLATES

(The electron micrographs in this study were made with the assistance of Mr C. C. Newton)

Plate 1

Fig. 1. *Salm. typhi*, microcyst, showing total absence of flagella. Electron micrograph, gold-shadowed. × 30,000.

Fig. 2. *Pr. vulgaris*, germinating microcyst, showing two sub-polar flagella emerging from the cell wall. Note the appearance of collars. Electron micrograph, gold-shadowed. × 16,000. (See also Fig. 1, which is traced from this micrograph.)

Fig. 3. *Salm. typhi*, germinating microcyst. One sub-polar flagellum is partly grown, and the base is surrounded by a collar. Other flagella are commencing to emerge. Electron micrograph, gold-shadowed. × 27,000. (See also Fig. 2, tracing.)

Fig. 4. As fig. 3, silver impregnation. × 3000.

Fig. 5. *Bact. coli*, germinating microcyst. Two polar flagella are just emerging through the cell wall. A basal structure (blepharoplast?) is seen. Electron micrograph, gold-shadowed. × 20,000.

Plate 2

Figs. 6, 7. As fig. 5, polar flagella further developed. Electron micrograph. × 16,000.

Fig. 8. *Pr. vulgaris*, germinating microcysts with short, developing flagella. Silver impregnation. × 3000.

Figs. 9, 10. *Salm. typhi*, young vegetative cells with developing, sub-polar flagella. Silver impregnation. × 3000.

Fig. 11. *Ps. fluorescens*, germinating microcysts showing emergent flagella. Electron micrograph, gold-shadowed. × 16,000.

Plate 3

Figs. 12–15. *Ps. fluorescens*, germinating microcysts showing developing flagella. Silver impregnation. × 3000.

Fig. 16. *Bact. coli*, young vegetative cell with growing lateral flagellum. Electron micrograph, gold-shadowed. × 16,000.

Figs. 17–19. *Salm. typhi*, young vegetative cells showing developing flagella. Silver impregnation. Figs. 17, 18 × 3000; fig. 19 × 5000.

Fig. 20. *Ps. fluorescens*, young vegetative cell with developing flagella. Electron micrograph, gold-shadowed. × 16,000.

Figs. 21, 22. *Ps. fluorescens*, young vegetative cells with emergent flagella at opposite pole. Silver impregnation. × 3000.

Plate 4

Fig. 23. As figs. 21 and 22, electron micrograph, gold-shadowed. × 9,000.

Fig. 24. *Salm. typhi*, cell with fully developed flagella in characteristic arrangement. Electron micrograph, gold-shadowed. × 9,000.

Fig. 25. As fig. 24, silver impregnation. × 4,000.

Fig. 26. *Ps. fluorescens*, cells with fully developed flagella. Silver impregnation. × 3000.

Fig. 27. *Salm. typhi*, maturing microcysts and senescent vegetative cells, showing cast-off and broken flagella. Electron micrograph, gold-shadowed. × 9,000.

Fig. 28. *Ps. fluorescens*, wall of partially disrupted cell, showing a group of basal granules (blepharoplasts?) at the bases of the flagella. Electron micrograph, gold-shadowed. × 12,000.

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The Development of the Surface Structures in Dividing Bacteria

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SUMMARY: The cell wall of growing Gram-negative bacteria is formed mainly, or entirely, at one pole of the cell. At this point the cell wall is soft and transparent to the electron beam, and there is a concentration of stainable material in the underlying cell membrane. A similar 'growing point' occurs at the point of cell division. In dividing bacteria, the portion of the surface which bears the flagella is retained in its entirety by one daughter cell, the other daughter cell grows new flagella.

In the course of an investigation of the growth of bacterial flagella (Bisset & Hale, 1951) it was observed that the flagella tended to develop at one pole of the cell in the process of germination from the microcyst stage. It was also observed that, in dividing cells of *Salmonella typhi*, one daughter cell was usually flagellated while the other was not. This phenomenon was further examined, and observations were made upon *Proteus vulgaris* and *Pseudomonas fluorescens*.

METHODS

Electron microscopy and silver impregnation stains for photomicrography were used, exactly as in the foregoing study (Bisset & Hale, 1951).

OBSERVATIONS

Preparations were made of young cultures from 2 to 24 hr. old. When the original inoculum consisted of non-flagellated microcysts (Bisset, 1950) cell division commenced shortly after germination and was active after 3 or 4 hr.

Salmonella typhi. Dividing cells first elongated considerably, and in this condition it was already apparent that almost all flagella were confined to one-half of the cell (Pl. 1, figs. 1–3). When division was complete all the flagella remained on one daughter cell, the other being quite devoid of flagella or just commencing to produce them (Pl. 1, figs. 4, 5).

Proteus vulgaris. This bacterium divided in a similar manner, but there was a significant difference from the condition in *Salm. typhi.* The original flagella were retained upon one half of the growing cell, but new flagella began to develop upon the other half before division was completed. Thus in a bacterium which was on the point of division, or had just divided, one daughter cell usually had much longer flagella than the other (Pl. 2, figs. 6–9).

Growing points. The conclusion from these observations upon the behaviour of the flagella in dividing bacteria is that the cell wall and flagella-bearing structures are produced at one pole of the cell. This was borne out by the appearance of the cell wall in electron micrographs. Nearly always the pole distant from the flagella was soft and transparent in appearance. This can be seen in *Salm. typhi* (Pl. 1, figs. 2, 4), but is more obvious in *Proteus* (Pl. 2, figs. 6, 10). When unfixed bacteria in this condition were lightly stained with an aqueous solution of Giemsa, one pole usually stained more intensely blue than the remainder of the organism (Pl. 2, fig. 11). It may reasonably be assumed that this stainable material represents a concentration of secretory nucleic acids, which are responsible for the formation of the cell wall. A similar but rather less intense concentration occurred at the points of cell division, corresponding to the areas of secretion of cell-wall material indicated in a previous work (Bisset, 1948).

Pseudomonas fluorescens. The development of the flagella in this species was naturally somewhat different from the condition in the bacteria described above. The flagella are arranged in a tuft at one pole, but occasionally, in germinating microcysts, flagella may develop at the opposite pole before the initiation of cell division (Bisset & Hale, 1951). This may possibly indicate that the polarity of growth of such cells is not yet established. More usually the flagella were confined to one pole until division was well advanced, or even completed (Pl. 3, figs. 12–16). The growing point of *Ps. fluorescens* was not noticeably transparent to the electron beam, perhaps because it was seldom far removed from the flagella-bearing condition, but the underlying, stainable, polar material was well seen (Pl. 3, fig. 17).

DISCUSSION

The foregoing observations provide additional evidence in support of the author's contention (Bisset, 1950) that the cell wall of bacteria is a dead structure, which does not grow but is secreted. It now appears that this secretion occurs mainly, if not exclusively, at two places, the point of division of a dividing cell (Bisset, 1948) and at one pole of a growing cell (Fig. 1). The new cell wall in process of formation is soft and transparent to the electron beam, presumably because it is thin. The concentration of stainable material in the growing point probably represents an area of secretory activity with a high proportion of

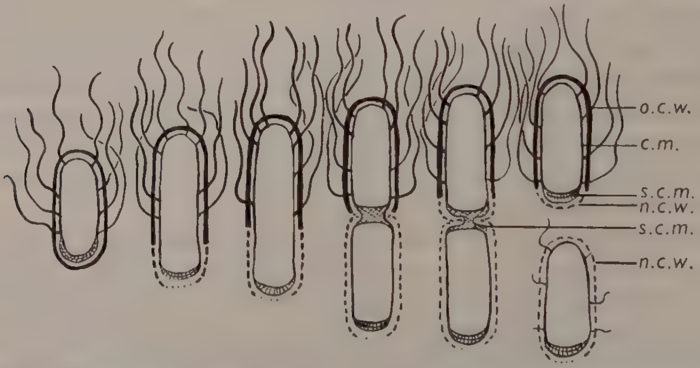
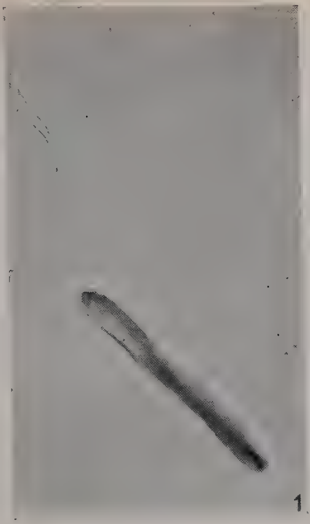


Fig. 1. The development of the cell wall in a bacterium with peritrichous flagella: *o.c.w.* old cell wall; *c.m.* cell membrane; *s.c.m.* secretory cell membrane; *n.c.w.* new cell wall.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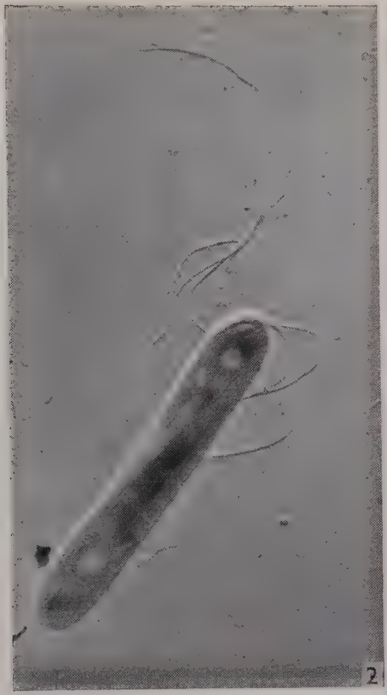
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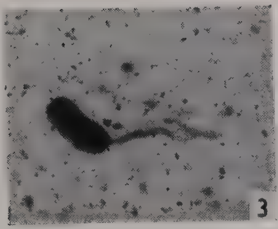
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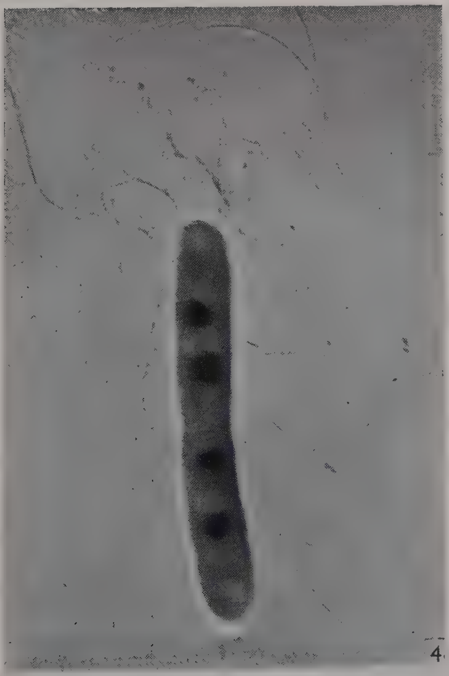
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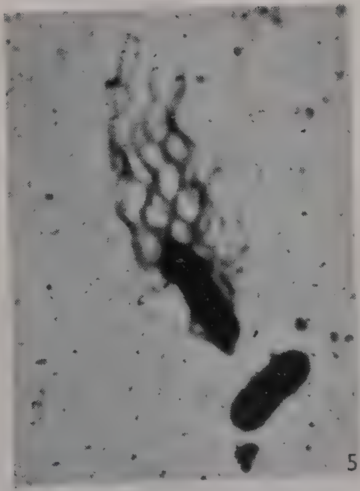
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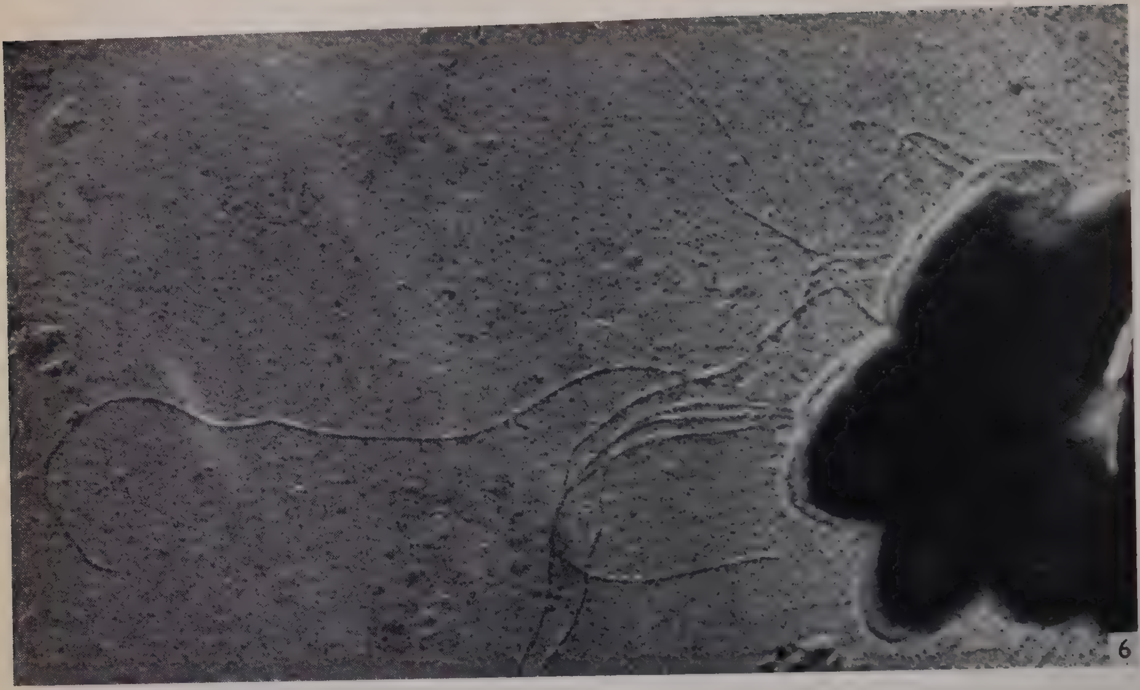
Figs. 1–5

K. A. Bisset—Surface structures in dividing bacteria. Plate 1



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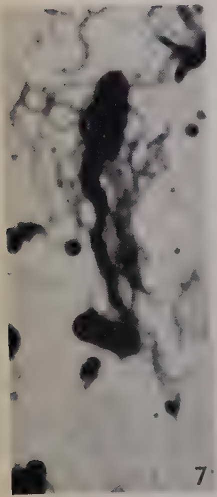

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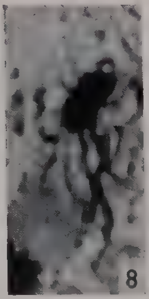

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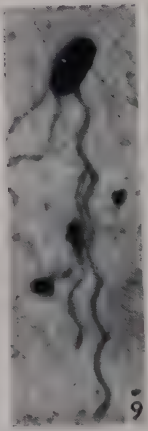

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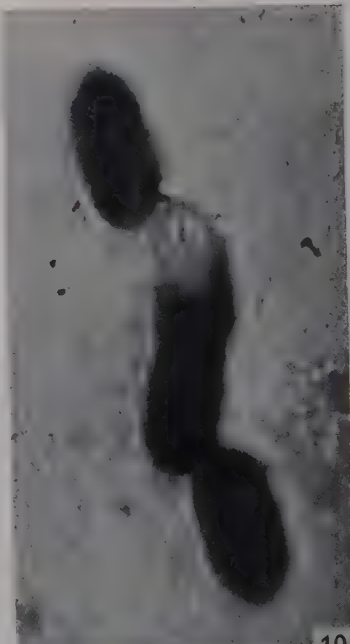

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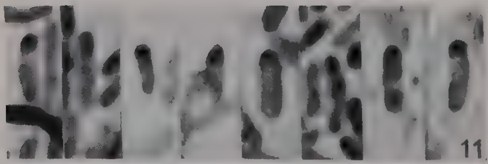

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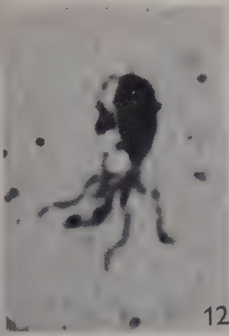
Figs. 6–11

K. A. Bisset—Surface structures in dividing bacteria. Plate 2



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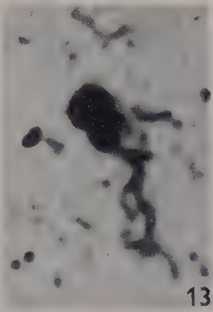

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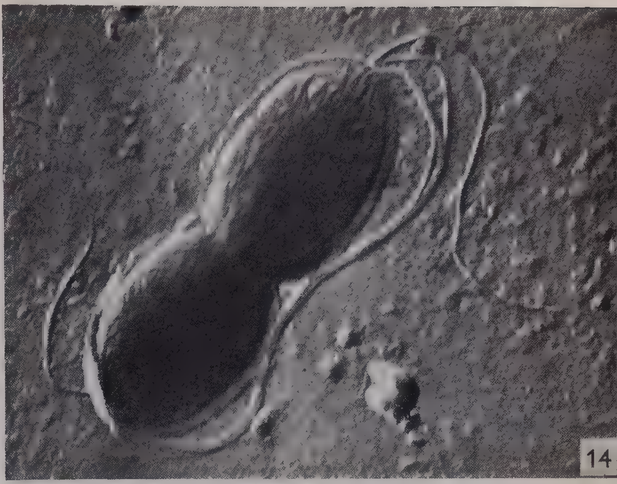

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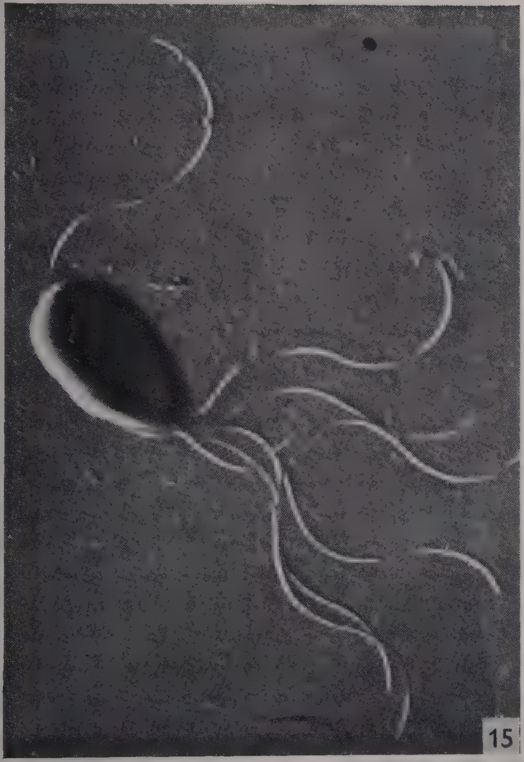

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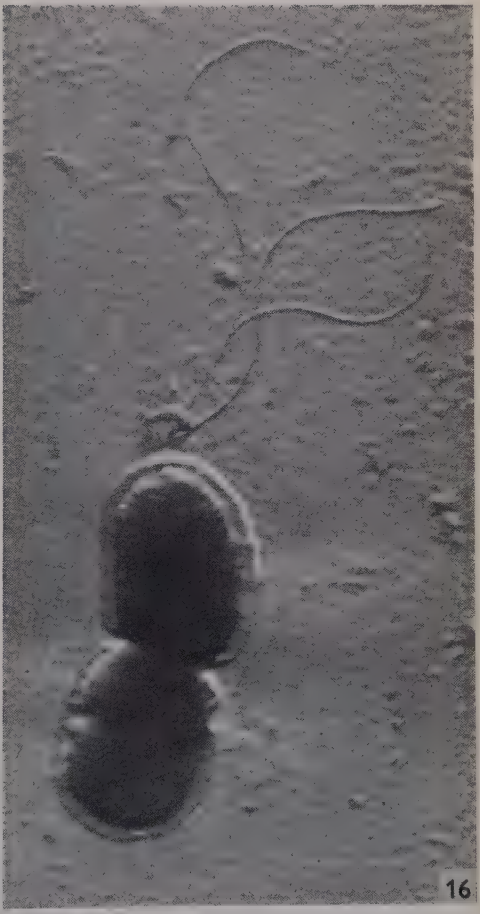

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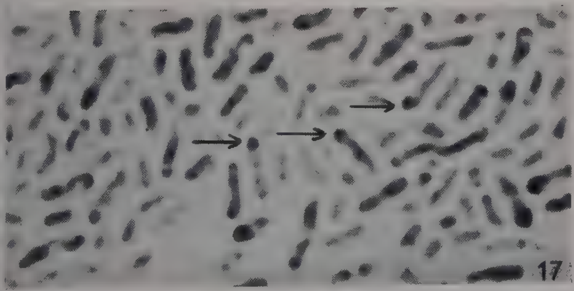

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Figs. 12–17

K. A. Bisset—Surface structures in dividing bacteria. Plate 3

nucleic acids. The growing point of the 'older' daughter cell is apparently derived from the point of division, but the 'younger' daughter cell retains the original active pole, at least in the case of *Proteus*, although *Pseudomonas* may behave in a slightly different manner, as is explained below.

The flagella behave exactly as though they were firmly attached to the cell wall, and their positions, relative to each other, remain constant as the cell enlarges. This is to say their relative distances do not increase, as they would if the whole cell wall increased in area. The new cell wall, at the growing end of the cell, does not begin to produce flagella until it has achieved a certain degree of maturity. In the case of *Proteus* it may do so fairly soon after its formation; in the case of *Salm. typhi* the non-flagellated period is much longer. The behaviour of *Ps. fluorescens*, in this respect, is conditioned by the polar position of the flagella, but the author's interpretation is that, most usually, the flagella do not begin to develop upon the growing tip, but do so only after elongation of the cell at this point has ceased, so that the growing tips of both cells are derived from the new point of division. This constant alteration in polarity may be reflected in the occasional production of flagella at both ends of germinating microcysts.

The behaviour of these surface structures at cell division suggests that bacteria do not divide by simple fission, but that each cell produces a bud of a size which is often, although not always, equal to that of the parent cell.

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EXPLANATION OF PLATES

(The electron micrographs were made with the assistance of Miss C. M. F. Hale and Mr C. C. Newton.)

Plate 1

Figs. 1, 2. *Salm. typhi.* Young bacteria on the point of division. Flagella confined to one end. Electron micrograph, gold-shadowed. Fig. 1, × 5,500; fig. 2, × 9,000.

Fig. 3. As figs. 1 and 2. Silver impregnation. × 3,000.

Fig. 4. *Salm. typhi.* Dividing cell, all flagella are confined to one daughter cell. Observe short, growing flagella at one pole and transparent growing point at the other. Electron micrograph, gold-shadowed. × 7,000.

Fig. 5. *Salm. typhi.* Bacteria immediately after division. One daughter cell is flagellated, the other bears only rudimentary flagella. Silver impregnation. × 6,000.

Plate 2

Fig. 6. *Pr. vulgaris.* Dividing bacterium. The growing point is clearly seen on the right, and this cell has shorter flagella than the left-hand daughter cell, which has longer flagella and no growing point. Electron micrograph, gold-shadowed. × 2,000.

Figs. 7, 8. *Pr. vulgaris.* Bacteria growing and dividing. Long flagella upon one half of the cell, short or none on the other. Silver impregnation. × 3,000.

Fig. 9. *Pr. vulgaris.* Recently divided bacteria. One daughter cell has many well-developed bacteria, the other has very few, and these much shorter. Silver impregnation. ×3000.

Fig. 10. *Pr. vulgaris.* Actively growing bacterium with transparent growing point (flagella not well shown) between resting bacteria without differentiated poles. Electron micrograph, gold-shadowed. ×8,000.

Fig. 11. *Salm. typhi.* Growing points. Aggregations of stainable material at one pole of the cell and at the point of division. Giemsa. ×3000.

PLATE 3

Figs. 12–15. *Ps. fluorescens.* Dividing bacteria with fully developed tufts of flagella at one pole and single, growing flagella at the other. Figs. 12, 13, silver impregnation, ×3000; figs. 14, 15, electron micrographs, gold-shadowed, ×16,000.

Fig. 16. *Ps. fluorescens.* Dividing bacterium with flagella at only one pole. Electron micrograph. ×16,000.

Fig. 17. *Ps. fluorescens.* Growing points. Aggregations of stainable material at one pole of the bacterium, and at the point of division. Giemsa. ×3000.

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SKINNER, F. A. (1951). *J. gen. Microbiol.* 5, 159–166.

A Method for Distinguishing Between Viable Spores and Mycelial Fragments of Actinomycetes in Soils

BY F. A. SKINNER

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SUMMARY: When shaken in suspension, vegetative actinomycete mycelium breaks into a large number of viable fragments which are killed when shaking is prolonged. Spores do not break up into smaller particles and are more resistant to shaking than vegetative fragments. Suspensions of vegetative mycelium may be distinguished from spores by the manner in which the viable count varies with time of shaking. While it is not possible to estimate the numbers of spores and of vegetative particles in mixed suspension the predominating form may be identified. Soil samples from Broadbalk field had a high count of actinomycetes which were almost certainly present as free spores.

Estimates of microbiological activity in soils are usually made by determining the numbers of micro-organisms present by the plating technique. In general, this method is satisfactory for counting unicellular organisms but it cannot be used to give an accurate estimate of the extent of actinomycete (*Streptomyces* spp.) growth. Since a colony may originate either from a spore or from a fragment of mycelium it is therefore clear that the numbers of colonies appearing on plates give no information about the activity of these organisms at the time of sampling (Waksman, 1927).

Brierley, Jewson & Brierley (1927) showed that in shaken soil suspensions the viable count of actinomycetes increased steadily with the shaking time. This result may have been caused either by a gradual separation of particles from disintegrating soil crumbs or by a progressive breaking-up of mycelium. Tests in this laboratory have shown the latter explanation to be more probable. A possibility to be considered is that a gradually rising count might not be obtained if the actinomycetes were present in the soil only as spores at the time of sampling. The following experiments were made to study the effect of shaking on suspensions of vegetative mycelium and of spores and to examine the possibility of distinguishing by these means between these two kinds of viable particles.

MATERIALS AND METHODS

Organism used. All experiments other than those on soil suspensions were made with a pure culture of a single species isolated from Broadbalk soil. It was related to *Streptomyces albidoflavus* (Rossi Doria) comb. nov.

Preparations of suspensions. Suspensions of vegetative mycelium were prepared by growing the actinomycete in the bottles in which they would eventually be shaken, i.e. a whole culture was shaken for each experiment. Ten ml. portions of nutrient broth (NaCl, 5·0 g.; Hopkin & William's peptone, 10·0 g.; Lab-Lemco, 3·0 g.; Marmite, 1·0 g.; distilled water, 1 l.; adjusted to pH 7·2) were added to 1 oz. (28 ml.) culture bottles with sand to assist in breaking up the mycelium. Bottles were then plugged and autoclaved for 15 min. at 15 lb./sq.in. Each sterile bottle received one drop of a heavy spore suspension and was incubated at 25°. Cultures prepared in this way showed good bottom vegetative growth consisting of a large number of minute colonies, in a few days. Before shaking, the plug was replaced by a sterile metal screw-cap and the bottle agitated gently by hand for 2 min. to render the suspension reasonably homogeneous. An initial sample to give the count at zero time was then taken. Shaking was performed by a reciprocating machine in which the bottles moved through a vertical distance of 9 cm. at a rate of about 265 strokes/min. Samples (0·5 ml.) were taken from the bottles at intervals during the experiment, for dilution in sterile saline (0·75 % (w/v) NaCl) and plating. Counts were made on three replicate plates for each sample after 7 days' incubation.

Spore suspensions were made by washing the sporulating surfaces of 10-day-old slope cultures of the actinomycete on Waksman's starch-tryptone agar, with a detergent consisting of a 0·1 % (v/v) solution of Calsolene Oil HS (I.C.I. (Pharmaceuticals) Ltd.) in distilled water. Washings were bulked and 10 ml. portions transferred to empty sterile culture bottles. These suspensions were shaken and sampled in the same way as those of vegetative mycelium.

EXPERIMENTAL

As a preliminary experiment (Exp. 1), 250 ml. of a suspension of vegetative mycelium were shaken by hand in a Roux bottle with 25 g. sand. The viable count increased with time of shaking; rapidly at first and then more slowly, indicating the progressive fragmentation of pieces of mycelium. This result was similar to those obtained by Brierley *et al.* for soil suspensions. Their results could thus be explained by supposing the actinomycetes to be present in these soils in an active vegetative state. It should be noted, however, that the gradual rise in count in the above experiment occurred in the presence of a considerable quantity of sand, whereas in the experiments of Brierley *et al.* no sand was present other than that in the soil samples. Further experiments were made to determine whether vegetative mycelium would be broken up in this way when suspensions were shaken without abrasive. Mechanical shaking was employed to standardize technique and to make possible the treatment of several suspensions at the same time.

Mechanical shaking of vegetative mycelium

Exp. 2. Four 9-day-old vegetative cultures were prepared, the bottles containing sand in the quantities shown: A, no sand; B, 1 g. sand; C, 2 g. sand; D, 5 g. sand. These cultures were shaken for 1 hr. and sampled as previously described. The result for culture A showed that the shaking of mycelium without sand could cause a gradual breaking-up of the fragments (Fig. 1). A similar increase in the number of viable particles was found for B and C. It was evident that though sand assisted the fragmentation of mycelium considerably, its presence was not essential for the production of a gradually rising colony count of the type found by previous workers (Brierley *et al.*, 1927) for soil suspensions. Clearly, their results were consistent with the view that actinomycetes were existing in a vegetative state in those soils examined. A very different result was obtained for culture D. The colony count increased rapidly at first, to reach its highest value at the 15 min. sampling time. Numbers then fell sharply as shaking was continued, demonstrating rapid killing of the fragments. Curran & Evans (1942) found that bacterial spores were killed when shaken in suspension with inert particles, killing being evident from the beginning of the experiment. A similar result for vegetative cells was found by King & Alexander (1948). It was probable, therefore, that some fragments of mycelium were killed from the beginning in all suspensions containing sand but that the rate of killing was masked by the more rapid formation of new fragments from the larger pieces of mycelium. After shaking for 15 min. it was clear that the rate of killing exceeded the rate of formation of new particles.

The result for culture D was confirmed by an additional experiment (Exp. 3) in which a 10 ml. vegetative culture was shaken with 5 g. sand. A larger number of samples (each of 0·1 ml.) were taken over a shaking period of 90 min., to provide adequate data on the decline in numbers. The points obtained by plotting logarithms of the colony counts for samples taken after the 15 min. sampling time, against the shaking time, lay about a straight line. This showed that the resultant of the rates of killing and of formation of new fragments was an exponential decline. Both Curran & Evans (1942) and King & Alexander (1948) observed that bacteria were killed exponentially when shaken in suspension with glass beads. Since there is no evidence to suggest that small pieces of mycelium will be destroyed according to some different mathematical law it may be inferred that there was practically no formation of new fragments after shaking for 15 min. Thus, from these experiments, it appears that the initial effect of shaking on suspensions containing vegetative growth is to break up the pieces of mycelium with a consequent increase in the number of viable particles. Prolonged shaking in the presence of sand brings about a progressive killing of these fragments.

Mechanical shaking of spore suspensions

Exp. 4. Two 10 ml. portions of spore suspension (A, no sand; B, with 5 g. sand) were shaken as described. Counts for both suspensions were initially high and remained fairly constant during the whole shaking period (Table 1). There was no evidence that the spores could break up into smaller viable particles or that prolonged shaking even with 5 g. sand had much effect on their viability (cf. Exps. 2 and 3).

These differences between the results for spore suspensions and those for vegetative suspensions were very marked. In the former case, there was little variation among colony counts for different times of shaking, at least during the first 15 min. of the experiments. Whenever the actinomycete was present in the mycelial condition, however, then evidence of fragmentation of that mycelium was given by a rising count over the same shaking period. A con-

Table 1. *Suspensions of actinomycete spores shaken mechanically*

(Dilution: 1/20,000.)

Time of shaking (min.)	Colonies/plate (mean of three replicate plates)	
	A (no sand)	B (5 g. sand)
0	443·3	328·0
5	322·0	468·0
15	296·0	448·7
30	319·3	412·7
60	296·7	371·3

venient way of representing such results without regard for the actual numbers of viable spores or fragments in any experiment, is to take the ratio of the count at some particular time (e.g. 15 min.) to the initial count. This ratio is large for suspensions of vegetative mycelium but approximates to unity for suspensions of detached spores. Ratios derived from all the experiments are given in Table 2.

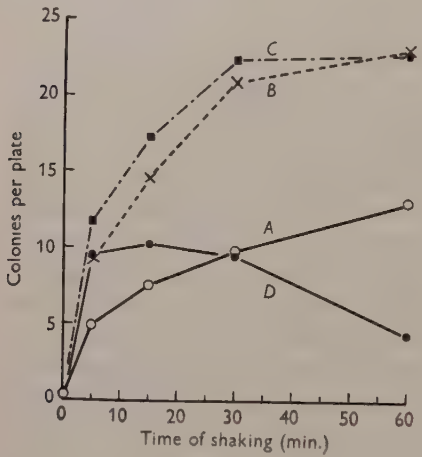

Fig. 1

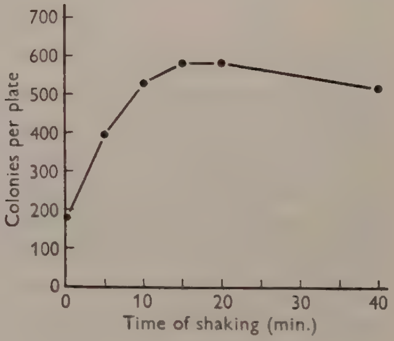

Fig. 2

Fig. 1. Suspensions of pieces of vegetative actinomycete mycelium shaken mechanically (Exp. 2). A, without sand; B, with 1 g. sand; C, with 2 g. sand; D, with 5 g. sand. Plating dilution: 1/20,000.

Fig. 2. Suspension of actinomycete soil culture shaken mechanically with 5 g. of sand (Exp. 10). Plating dilution: 1/250,000.

Suspensions of soil

In view of these results it seemed probable that information about the state in which actinomycetes were present in soil might be obtained by shaking soil suspensions in the manner described, and observing the way in which the count of *Streptomyces* colonies varied with the shaking time.

Experiments were made with soil samples taken from the following three plots of Broadbalk field (permanent wheat): plot 2, receiving 14 tons of dung/

acre/year; plot 3, untreated; plot 7, receiving complete minerals (3·5 cwt. superphosphate, 2 cwt. potassium sulphate, 1 cwt. sodium sulphate, 1 cwt. magnesium sulphate and 4 cwt. ammonium sulphate/acre/year).

Table 2. *Ratios between colony counts at 15 min. sampling times and initial counts*

Experiments on suspensions of	Exp.	Suspension	Ratio
(*a*) Vegetative mycelium	1		23·2
	2 (Fig. 1)	A	28·1
		B	34·8
		C	58·0
		D	50·9
	3		19·3
	8 (Table 3)	A	49·4
		B	97·5
	9	A	97·2
		B	43·1
		C	19·7
(*b*) Spores	4 (Table 1)	A	0·7
		B	1·4
	11		1·3
(*c*) Soil	5	A	0·9
		B	0·8
	6		1·5
	7		1·8
(*d*) Soil culture	10 (Fig. 2)		3·2

Exp. 5. Five g. of sieved plot 2 soil were added to 10 ml. sterile saline (suspension A): a duplicate suspension (B) contained in addition 5 g. sand. These were shaken and sampled in the usual way. Plates were examined microscopically to distinguish *Streptomyces* colonies from those of other organisms. A high initial count of *Streptomyces* spp. was found for each suspension. These counts varied little during the experiment, though a slight fall occurred between the 20 and 40 min. sampling times in the suspension shaken with sand. It was therefore considered probable that in this soil actinomycete spores were present in abundance but that there was little if any vegetative mycelium. Similar results were found for soils taken from plots 3 (Exp. 6) and 7 (Exp. 7).

Detection of small quantities of vegetative mycelium in suspension

The experiments on pure cultures of actinomycete mycelium (Exps. 2 and 3) were made with suspensions containing considerable amounts of vegetative growth (*c*. 10 mg. dry wt./10 ml. culture). However, it is unlikely that such a large quantity of mycelium would ever be found in a suspension of natural soil of the concentration used in Exps. 5–7. It was therefore considered necessary to determine whether mycelial fragments in very low concentration would be broken up sufficiently quickly on shaking to give the rapid initial rise in numbers so characteristic of heavier suspensions.

Exp. 8. Samples (1·0 ml.) of a 2-day-old broth culture of vegetative mycelium were diluted 1/10 and 1/100 in saline. In the latter suspension only a very few minute flecks of mycelial growth could be seen. Ten ml. portions of both suspensions were shaken with 5 g. quantities of sand. The results (Table 3) showed the same rise and fall in numbers as was previously found for heavier mycelial suspensions shaken with this quantity of sand; so it was concluded that mycelium could be detected by this method even when present in very small quantities.

Table 3. *Suspensions of vegetative actinomycete mycelium shaken mechanically*

Time of shaking (min.)	Colonies/plate (mean of three replicate plates) Vegetative culture diluted: A, 1/10 in saline	B, 1/100 in saline
0	2·7	1·3
5	151·0	180·0
15	133·3	126·7
30	69·7	37·0
60	12·7	3·3
Plating dilutions	1/2000	1/200

The effect of soil particles on the fragmentation of mycelium

No evidence was found for the presence of mycelium in suspensions of natural soils. The suspensions used, however, contained large amounts of soil (5 g./10 ml.) and such a high concentration of soil particles might prevent the breaking of any mycelial fragments present, thereby giving a result apparently indicating the absence of mycelium. The following experiment (Exp. 9) was made to determine the effect of different amounts of soil on the rate of break-up of vegetative fragments. Three suspensions, each consisting of 10 ml. of a 1/100 dilution of vegetative culture in saline, were prepared. Sterile soil was then added to the bottles in the following quantities: A, no soil; B, 0·5 g. soil; C, 5·0 g. soil: each bottle also contained 5 g. sand. Shaking and sampling were performed in the usual way. The results were characteristic of those always found for vegetative suspensions, though it was clear that a high concentration of soil particles (as in C) diminished the maximum count considerably either by preventing fragmentation through restricting movement of the sand grains, or by increasing the death-rate. It was concluded that in spite of the presence of a large amount of soil in suspension, very small quantities of mycelium in that suspension could still be detected by the shaking technique.

The effect of shaking a soil culture of Streptomyces *sp. containing both spores and vegetative mycelium*

It has been shown that suspensions of vegetative actinomycete mycelium can be distinguished from those of spores. It has also been shown that actinomycetes were probably present as spores in the soils examined. In some soils, however, it is to be expected that both spores and vegetative mycelium will be present together, in which case shaken suspensions of these soils should give results different from those found for Broadbalk soils. An experiment was made on a pure soil culture known to contain both vegetative mycelium and spores of the actinomycete to determine whether these two kinds of viable particles could be distinguished when present in the same suspension.

A soil culture was prepared as follows: 30 g. of sieved, air-dried Broadbalk soil (plot 3) were placed in a Petri dish and autoclaved for 1 hr. A suspension of spores in 12 ml. nutrient broth was distributed over the soil surface. After incubation for 7 days the actinomycete had made good growth, aerial mycelium being visible on many of the soil crumbs. Five g. of this soil culture were added to 10 ml. saline in a shaking bottle with 5 g. sand and treated in the usual way (Exp. 10). The results (Fig. 2) yielded a curve which showed some features characteristic of curves derived both from spore and mycelial suspensions. The high initial count was almost certainly caused by a rapid release of spores from the soil particles, and the rise in count during the first 15 min. of shaking indicated the break-up of vegetative mycelium. It was considered possible, however, that this rise in count might have been caused partly by a gradual breaking-up of spore aggregates and sporogenous aerial hyphae. To determine the rate at which such aggregates would be disrupted on shaking a further experiment was made. The aerial growth was removed from an agar slope culture of the actinomycete by dragging a wire loop across the surface, and added to 10 ml. saline with 5 g. sand in a shaking bottle (Exp. 11). On shaking, a high initial count was obtained. This was followed by a slight rise in count after shaking for 5 min. and then by a fall. The occurrence of the highest count so early in the experiment showed that spore aggregates were broken up very easily. The subsequent decline in numbers was not so readily explained, but probably it resulted from the destruction of undifferentiated aerial hyphae which formed part of the surface growth removed by the wire loop. In view of these results it was thought probable that the rising count obtained in the soil culture experiment was due almost entirely to the breaking-up of mycelial fragments. It seemed clear from these results that the shaking technique described could be used to analyse mixed suspensions of spores and vegetative fragments.

DISCUSSION

The evidence presented suggests that species of *Streptomyces* were present in Broadbalk soils as spores at the time of sampling. A similar conclusion for Indian soils was reached by Subrahmanyan (1929) who claimed to be able to distinguish between colonies of conidial and mycelial origin. Whether actinomycetes are usually present in this dormant state in soils has not yet been established, but it is evident that vegetative development must sometimes occur even though such periods of activity may be of short duration. Results (Exp. 9) have shown that vegetative growth could be detected in soil culture by the use of shaken suspensions. Support has also been given (Exp. 1 and 2) to the opinion that vegetative mycelium was present in soils examined by other workers (Brierley *et al.* 1927). Other methods such as the direct observation of glass slides which have been buried in soil may be used to study the development of actinomycetes in soil culture (Jensen, 1943), but it is an advantage to be able to detect the presence of vegetative mycelium and spores at any instant of time in natural soils. The shaking technique described may be used to obtain such information and so assist in the solution of a complex problem. It is clear, however, that any attempt to make these methods useful for quantitative estimations should be delayed until further investigations have been made on the behaviour of other species of *Streptomyces* in shaken suspensions.

I wish to express my thanks to the Agricultural Research Council for the grant which made this work possible, to Dr H. G. Thornton, F.R.S., and Dr Jane Meiklejohn for helpful advice and criticism, and to Miss Mabel Dunkley for assistance in preparing the manuscript.

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The Measurement of the Aeration of Culture Media

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SUMMARY: The aeration of culture media can be measured in terms of ϕ_L (defined below) which is dependent on rate of air flow, agitation, etc. Two methods of measuring ϕ_L are given, based on the polarographic estimation of dissolved oxygen. For penicillin and streptomycin fermentations there is a correlation between the amount of antibiotic produced and ϕ_L.

Recent advances in industrial stirred and aerated fermentations have emphasized the need for a suitable technique to measure aeration in terms of the oxygen available to the organism rather than in terms of the volume of air which is passed through the medium. In the case of submerged cultures, the organism is dependent upon the oxygen dissolved in solution. In order to assess the efficiency of the aeration it is therefore necessary to compare the rate of solution of oxygen into the culture medium with the rate of consumption of oxygen by the organism.

Determination of the oxygen demand

The Warburg apparatus is usually used for measuring oxygen demands, but in the present work it was found convenient to use a polarographic method first used by Petering & Daniels (1938). The culture is saturated with air and then the air supply is cut off. The concentration of dissolved oxygen in the culture medium decreases steadily from the saturation concentration to zero as the organism consumes the oxygen. A typical determination is shown in Fig. 1, from which it will be seen that the graph of the concentration of dissolved oxygen against time is linear, i.e. the oxygen demand is of zero order with respect to time and oxygen concentration over the period of measurement. This behaviour was always found with the organisms studied, and in the following theory it is assumed that the oxygen demand at a particular moment is of zero order. The polarographic measurements required only a few minutes so that no significant growth occurred during the measurement. The oxygen demand of the culture varies comparatively slowly with time as growth proceeds, and by measuring the oxygen demand of samples taken at various times throughout the culture period it is possible to draw the curve connecting oxygen demand with the time after the inoculation of the medium.

It has many times been noted (for a review see Tang, 1933) that the oxygen demand of many organisms is of zero order although only above a critical oxygen tension. Below the critical oxygen tension, the oxygen demand becomes first order. The critical oxygen tensions found are very low. For instance, in the present work the critical oxygen tensions were too low to be apparent from curves such as those shown in Fig. 1, so that the assumption that the oxygen demand is of zero order is justified.

Determination of the rate of solution of oxygen

If a culture vessel is filled with water which has previously been freed from dissolved oxygen by gassing-out with nitrogen, then when the air supply to the vessel is switched on, the concentration of dissolved oxygen rises from zero to the saturation concentration (C_e) as shown in Fig. 2. The rate of solution of oxygen is given by the slope of the tangent to the curve, e.g. when $C=0{\cdot}5\,C_e$ the rate of solution is the slope of the dotted line. It will be clear that when the concentration of dissolved oxygen is at the saturation concentration, C_e, the slope of the tangent is zero. This is as expected, since once the water has become saturated with oxygen no more oxygen will dissolve from the air. Also, when the concentration of dissolved oxgyen is zero, the rate of solution of oxygen is the highest possible rate with the given vessel under the given conditions. This value of the rate of solution will be called the 'maximum solution rate'.

It is convenient to express the curve relating concentration of dissolved oxygen to the rate of solution of oxygen in the form of an equation. From the theory of gas absorption it would be expected that the rate of solution of oxygen is given by the equation

$$\frac{dC}{dt}=\phi_L(C_e-C), \tag{1}$$

where dC/dt is the rate of solution of oxygen at a concentration of dissolved oxygen C, and C_e is the saturation concentration of dissolved oxygen. dC/dt is the rate of solution of oxygen expressed as the rise in the concentration of dissolved oxygen. The total molecules of oxygen dissolving per minute from the air is given by $V(dC/dt)$, where V is the total volume of the liquid medium in litres and C is measured in molecules of dissolved oxygen per litre of medium.

From equation (1) it will be seen that when $C=C_e$, the rate of solution is zero and when $C=0$ the maximum solution rate is given by $\phi_L C_e$. It is found that equation (1) fits the experimental curves closely.

The theory of gas absorption enables the constant ϕ_L in equation (1) to be considered in terms of other variables, the most important of which are rate of air flow and agitation. The value of ϕ_L is proportional to the rate of air flow over a certain range. Hence ϕ_L is only constant in equation (1) if the rate of air flow is kept constant throughout the experiment. It is to be expected that agitation would increase the value of ϕ_L so that if the culture vessel is fitted with a stirring device this must be kept running at constant speed throughout the experiment. The value of ϕ_L will characterize the efficiency of aeration of a given vessel under fixed conditions of rate of air flow and agitation. Moreover, the effect of these variables on the aeration can be assessed by measuring the value of ϕ_L at various values of the rate of air flow or the rate of agitation, of which a rough measure is given by the speed of the stirrer.

The maximum solution rate, $\phi_L C_e$, can thus be controlled by adjusting the value of ϕ_L by varying the rate of air flow or the rate of agitation. It can also be controlled by altering the value of C_e. By Henry's Law, the value of the saturation concentration (C_e) is proportional to the partial pressure of oxygen in the gas phase. For instance, if the gas passed through the medium is changed from air containing 20% oxygen to pure oxygen, the value of C_e would be increased five times. Thus if the oxygen were passed at the same rate as the air, and if the agitation were kept constant, so that ϕ_L was the same in both instances, the value of the maximum solution rate would also be increased five times.

In the case of tall industrial vessels where the hydrostatic head is large, it might be expected that the pressure inside a bubble of air at the bottom of the vessel would be large enough to affect the value of C_e. This correction is necessary when values of C_e are being measured, but the value of the maximum solution rate, $\phi_L C_e$, is relatively insensitive to changes in the total pressure since an increase in the pressure on a bubble will decrease its surface area and this will decrease the value of ϕ_L. The two effects nearly cancel each other, and $\phi_L C_e$ is proportional to $P^{\frac{1}{3}}$ where P is the total pressure. Thus for moderate-sized vessels, the value of the maximum solution rate measured at any point in the vessel can be taken as correct over the whole vessel as far as the pressure effect is concerned.

Another effect which might be important for tall vessels is that as oxygen is consumed from a bubble, the value of the partial pressure of oxygen in the bubble will decrease, affecting C_e, and also the surface area of the bubble will decrease, affecting ϕ_L. Most aeration devices are so inefficient that this effect need not be considered. A treatment of the problem, however, has been given by Pattle (1950), who has produced very efficient aeration devices on a laboratory scale.

Aeration of a respiring culture

As mentioned above, the oxygen demands of cultures can frequently be expressed by the equation

$$\frac{dC}{dt} = k_0, \qquad (2)$$

where k_0 is a constant, the zero order oxygen demand.

If the contents of a culture vessel are aerated we have that (rate of increase in concentration of dissolved O_2) = (rate of solution) − (rate of consumption). Expressing this in terms of equations (1) and (2)

$$\frac{dC}{dt} = \phi_L(C_e - C) - k_0. \qquad (3)$$

As the concentration of dissolved oxygen rises, the rate of solution of oxygen will fall until eventually it reaches a value equal to the rate of consumption by the organism. When this is so, $dC/dt = 0$ and, representing the steady concentration maintained in the culture vessel by C_s, we have from equation (3) that

$$C_s = C_e\left\{1 - \frac{k_0}{\phi_L C_e}\right\}. \qquad (4)$$

Equation (4) shows that C_s is always less than C_e, but that $C_s \approx C_e$ when $\phi_L C_e$ is very much greater than k_0.

The conditions of inadequate aeration

It is often convenient to relate the yield of a metabolic product or the consumption of a medium constituent to the aeration rate. In this case the degree of aeration can be considered adequate when the maximum yield or consumption is obtained. However, this is not a fundamental measure, since the level of adequate aeration so defined will depend on the particular aspect of metabolism studied. Since the purpose of aeration is to supply the organism with the oxygen it needs, the adequacy of aeration can be unambiguously defined in terms of the relationship between the oxygen demand of the organism and the rate of supply of oxygen. A culture will be adequately aerated at a particular instant if the maximum solution rate $\phi_L C_e$ is greater than the oxygen demand of the organism. It is a consequence of the fact that the oxygen demand is of zero order that when the culture is adequately aerated, the oxygen demand will be independent of the rate of aeration.

When the culture is inadequately aerated, $\phi_L C_e < k_0$. This means that an organism which could consume oxygen at a rate k_0 is consuming oxygen only at a rate $\phi_L C_e$. The actual mechanism by which the organism consumes the oxygen at this rate will vary. It may be a rate-determining diffusion across the surface of, or into, the organism or else unsaturation of the enzymes reacting with the oxygen. In these cases, restoring the aeration to an adequate level within a short time should restore the oxygen demand to the original value k_0.

It is clear that maintaining the aeration at an inadequate level may alter the course of the metabolism, for instance by limiting growth or inducing a partial change to an anaerobic metabolism. In this case, restoring the aeration to an adequate level would result in an oxygen demand different from that of a similar culture which had been adequately aerated throughout. Thus in measuring adequacy of aeration, the reference state should be that of a culture which has been adequately aerated throughout.

EXPERIMENTAL

The concentration of dissolved oxygen was measured with the polarograph, using a dropping mercury electrode. The general technique used was similar to that of Petering & Daniels (1938). The method used is specific for dissolved oxygen. Air was supplied to the polarographic cell through a sintered glass disk in order to achieve a high value of ϕ_L. All samples taken contained enough inorganic salts to be polarographed directly, and no maxima on the wave were found. In exceptional circumstances it might be necessary to add supporting electrolyte to the sample before it was polarographed. When possible, the polarographic cell was kept in the same incubator as the fermenter; otherwise the cell was placed in a thermostat at the same temperature as the fermenter.

The potentiometer of the polarograph was fixed at $-0{\cdot}7$ V. which corresponded to the plateau at the top of the first oxygen wave. The current between the electrodes was taken as proportional to the concentration of dissolved oxygen. A dummy sample was taken to adjust the controls of the polarograph. The zero was established by bubbling nitrogen through the sample to remove all the dissolved oxygen and then adjusting the galvanometer deflexion to a suitably low value, using the zero-setting control. The sample was then saturated with air and a suitable deflexion of the galvanometer obtained by adjusting the galvanometer shunt. The instrument was then ready for use.

Measurement of oxygen demands

Samples of fermenting cultures were taken, placed in the polarographic cell and saturated with air. The air supply to the cell was then cut off and the concentration of dissolved oxygen measured at various times. When the sample had such a high oxygen demand that the value of C_s in the cell was much less than C_e, then the value of the current corresponding to C_e was obtained by inhibiting the oxygen demand with a trace of cyanide or other suitable inhibitor, and saturating again with air. The value of the zero was then determined to correct for any increase in current due to the inhibitor.

The polarograph measures the oxygen concentration in arbitrary units (see Lewis & Mackenzie, 1947). It is thus most convenient to express the results of oxygen demand experiments in terms of t_0, the time in minutes taken to reduce the concentration of dissolved oxygen from C_e to zero, a straight line being drawn through the experimental points, neglecting any tailing-off effect at low concentrations of dissolved oxygen.

Measurement of ϕ_L values

Two methods were employed, the gassing-out method based on equation (1) and the sampling method based on equation (4).

Gassing-out method. The vessel under test was filled with either the fermenting medium with the oxygen demand inhibited, or else with a solution of potassium chloride containing a trace of methyl red to eliminate the maximum on the oxygen wave. The vessel was arranged as for an actual fermentation. After a preliminary gassing-out with nitrogen, the aeration train was switched on and simultaneously a stop-clock was started. Throughout the determination the rate of air flow, usually measured with a calibrated capillary flowmeter, was kept as uniform as possible. At convenient intervals samples were removed and the concentration of dissolved oxygen determined polarographically. A graph of $\log (C_e - C)$ against t was drawn. By integrating equation (2) it can be shown that the slope of this plot is $-\phi_L/2{\cdot}303$, whence ϕ_L can easily be calculated.

The sampling method. The value of ϕ_L can be determined from equation (4) when C_s, C_e and k_0 are known. The values of C_e and k_0 can be obtained when the oxygen demand of a sample is measured. The value of C_s was obtained by taking a sample from an actual fermentation at a noted time, putting it quickly into the polarographic cell and then measuring the concentration of dissolved oxygen at various times. The graph of these results, extrapolated to the time at which the sample was taken, gave the value of C_s. The value of ϕ_L could then be calculated using equation (4). A correction was necessary when the partial pressure of oxygen in the gas passed through the culture vessel was different from that in the gas used in aerating the polarographic cell. To apply the correction, Henry's Law was assumed to be valid. It will be assumed as a convention that $C_e = 1$ for water in equilibrium with air at 1 atm. pressure.

RESULTS

A typical result of an oxygen-demand experiment is shown in Fig. 1. Usually good straight lines were obtained, but irregularities were sometimes observed, especially with penicillin fermentation samples. This was due to interference by the mycelium, and possibly the anti-foam agent, with the dropping of the mercury from the capillary. With some samples measurements were hardly possible. In general, it was found that the oxygen demand of penicillin and streptomycin fermentations was associated with the mycelium and that the medium itself usually had a negligible oxygen demand.

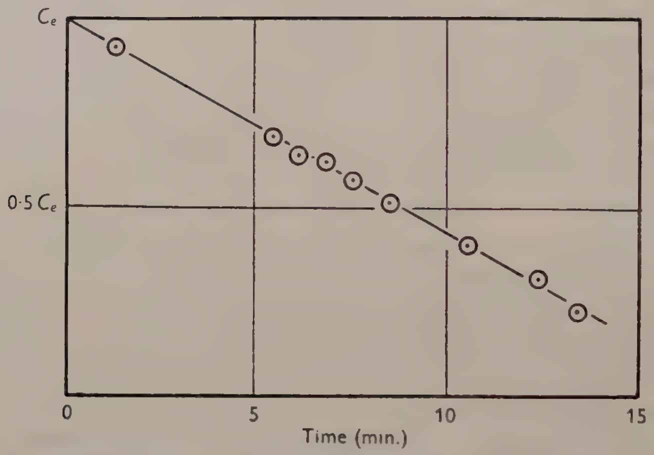

Fig. 1. Graph of concentration of dissolved oxygen against time for a culture initially saturated with air. C_e = saturation concentration of oxygen.

The results of a typical determination of ϕ_L using the gassing-out method is shown in Fig. 2 in which C and $\log_{10}(C_e - C)$ are plotted against t. Fig. 3 shows the result of a determination of ϕ_L using the sampling method.

Results obtained with the sampling method were much less accurate than those given by the gassing-out method. The difficulty inherent in the former method is that it is necessary for C_s to be large for a good extrapolation to be possible, but as C_s becomes larger, the value of $(C_e - C_s)$ becomes smaller and finally comparable with the experimental error in determining the concentrations of dissolved oxygen. The method can thus only be used when $C_s \approx \frac{1}{2} C_e$. This has to be arranged either by adjusting the air flow or by waiting until the oxygen demand has reached a suitable value. The advantage of the sampling method is that it can be used for examining fermentations in large production vessels which cannot be released for test purposes. The ordinary sampling points can be used during the routine fermentation providing that they yield a representative sample. By replication, the value of ϕ_L can be determined to within about 20 %. Greater accuracy is possible where polarographic measurements are subject to less irregularity, e.g. with yeast fermentations.

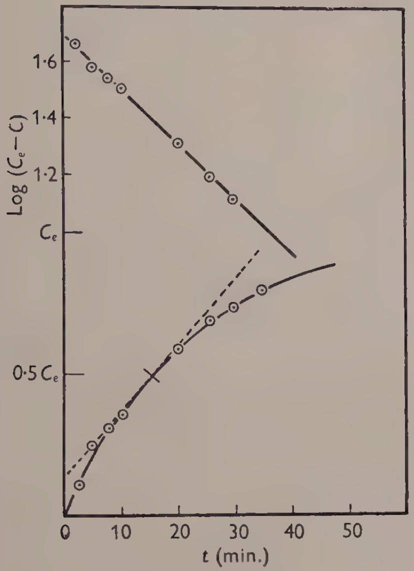

Fig. 2

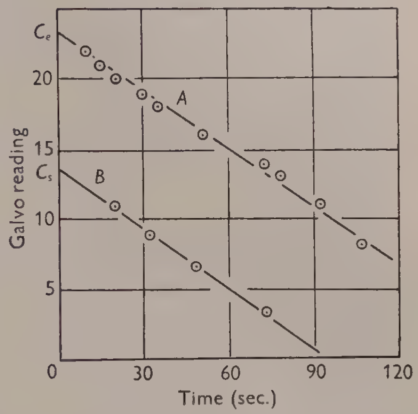

Fig. 3

Fig. 2. Graph of concentration of dissolved oxygen (C) against time for a vessel filled with potassium chloride solution initially oxygen-free. The upper graph is a plot of log $(C_e - C)$ against time for the same results. The ϕ_L value obtained from the upper curve is 0·045 min.$^{-1}$ and the full lower curve has been calculated using this value.

Fig. 3. Curve A: graph of concentration of dissolved oxygen (in arbitrary polarographic units) against time. Sample initially saturated with air. Curve B: sample from fermentation vessel removed at zero time and then placed in polarographic cell. The value of ϕ_L for this vessel is 0·85 min.$^{-1}$.

A cylindrical vessel 1 m. tall and 5 cm. in diameter, aerated through a 1 in. coarse sintered disk, was used for some laboratory tests, the gassing-out method being used. The results are shown in Fig. 4. The plot of ϕ_L against G, the rate of air flow, is linear over the range measured. Also it is shown in Fig. 4 that ϕ_L/G is independent of the volume of liquid in the vessel. This shows that the arbitrary convention of measuring aeration as volumes of gas/volume of medium/min. is meaningless.

Fig. 5 shows the effect of stirring on the ϕ_L value of a 50-gallon stirred fermenter, using the sampling method of measuring ϕ_L. It is clear that stirring has considerably increased the value of ϕ_L.

Fig. 6 shows a graph of the maximum titre, determined by bio-assay, produced in a streptomycin fermentation (*Streptomyces griseus* strain 5001 in salt glucose peptone medium) against $\phi_L C_e$ for various vessels in which the fermentation was conducted. Although the points show considerable scatter the correlation is convincing. When $\phi_L C_e > 1{\cdot}0$ no increase in titre is given by increasing the aeration. The value of the peak oxygen demand in these fermentations which occurred at the peak of growth corresponds to a value $\phi_L C_e = 1{\cdot}0$.

Fig. 7 shows a similar curve for penicillin fermentations (*Penicillium chrysogenum*, strain Q176, in a cornsteep liquor medium). This graph shows broadly the same characteristics as those obtained with the streptomycin fermentations.

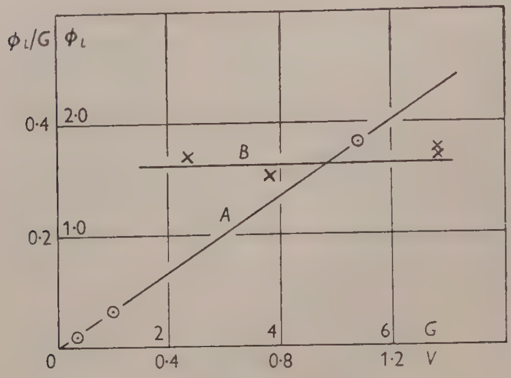

Fig. 4

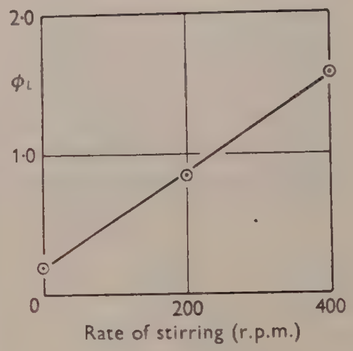

Fig. 5

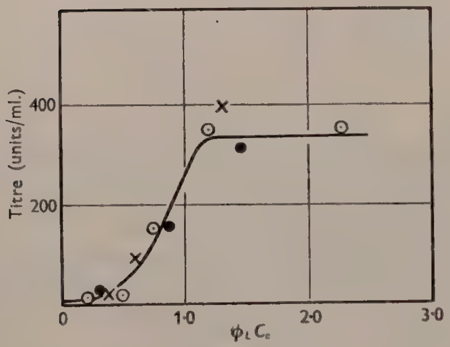

Fig. 6

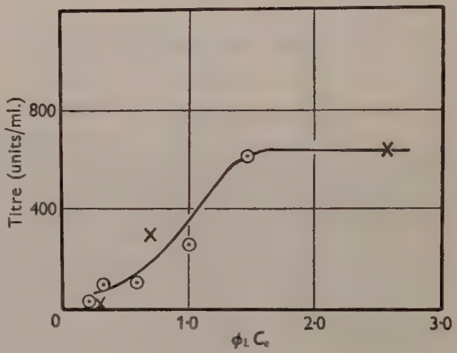

Fig. 7

Fig. 4. Curve A: graph of ϕ_L (in min.$^{-1}$) against G, the rate of air-flow in l./min. Curve B: graph of ϕ_L/G against V, the volume of liquid in litres.

Fig. 5. Graph of ϕ_L (in min.$^{-1}$) against rate of stirring for a 100-gallon vessel fitted with a stirrer. Rate of air flow constant at 15 cu.ft./min.

Fig. 6. Graph of maximum titre of streptomycin (at 75 hr.) against $\phi_L C_e$. ⊙—⊙, 5 l. stirred round-bottomed flasks; ●—●, 100 gallon vessel with stirrer. ×—×, 50-gallon vessel with stirrer. Each point is the average of five to ten experiments.

Fig. 7. Graph of titre, at 72 hr. after inoculation, of penicillin fermentations against $\phi_L C_e$. ⊙—⊙, 10 l. aspirators. ×—×, 50-gallon vessel with stirrer. Each point is the average of five to ten experiments.

The results shown in Figs. 6 and 7 were given by vessels in which the higher ϕ_L values were obtained either by a low rate of air flow and considerable mechanical stirring or else by fine aeration and no stirring. In spite of this, no correlation has so far been observed between titre and rate of stirring except in as far as the rate of stirring affected the value of ϕ_L. In this way the determination of ϕ_L values should allow a separation to be made of the two distinct effects of aeration and agitation. No effect of the size of the vessel is obvious in Figs. 6 and 7. This suggests that as far as aeration is concerned results obtained with small vessels may be used to predict the behaviour of much larger vessels. A similar result has been recorded for the case of yeast (Olson & Johnson, 1949).

DISCUSSION

The polarographic method of measuring oxygen demands shows clearly that dissolved oxygen is consumed by micro-organisms. The correlation between the *rate of solution* of oxygen and the antibiotic production of the organisms grown in different vessels shows that any effect due to some cells obtaining oxygen directly from air bubbles is very small. Moreover, the number of cells which could retain a contact with an air bubble sufficient to satisfy their oxygen demand must be small compared with the bulk of growth throughout the vessel.

The only previous reference found to the determination of ϕ_L values was published by Olson & Johnson whilst the present work was in progress. They determined the value of $\phi_L C_e$ by aerating a vessel filled with sodium sulphite solution containing copper ions. The concentration of dissolved oxygen in the liquid was always very close to zero, and the oxygen dissolving reacted quickly with an equivalent amount of sulphite ion to form sulphate. The value of $\phi_L C_e$ was obtained by measuring the amount of sulphite oxidized in a given time. Experiments in this laboratory have shown that the sulphite technique does not give the same value for $\phi_L C_e$ as the polarographic method. This is to be expected, since the rate of reaction of sulphite ions with oxygen is so fast as to affect the diffusion gradient across the air/liquid interface.

The fact that C_s is less than C_e has been tacitly assumed by many investigators. A clear demonstration, however, has already been given by van Goor & Jongbloed (1942) using a Warburg apparatus and estimating dissolved oxygen by a chemical method. They showed that the break-down from zero-order behaviour of the oxygen demand of brain-tissue of rats occurred at lower concentrations of dissolved oxygen than would be expected from the assumption that the oxygen tension in the medium was equal to the partial pressure of oxygen in the gas. Using the present methods on their graphs it seems that the ϕ_L value of the Warburg apparatus which they used was less than 0·2 min.$^{-1}$. This is rather low compared with many of the oxygen demands encountered in the present work, and confirms what is already known, namely, that the rate of transfer of oxygen from the gas phase of a Warburg apparatus to the liquid phase may be a limiting factor in measuring rates of oxygen uptake.

Certain general conclusions may be drawn from Figs. 6 and 7. The fact that the titre becomes independent of the rate of solution of oxygen above the value corresponding to the peak oxygen demand is due to the fact that the oxygen demand is of zero order. When the culture is adequately aerated, the oxygen demand, and thus the aerobic metabolism, is independent of the rate of aeration. When the culture is inadequately aerated, the rate at which oxygen is available is limited by the rate at which oxygen dissolves into the medium. Hence, if the antibiotic is produced in association with an aerobic process, the rate of antibiotic formation will become dependent on the aeration.

It is interesting that the titre falls off so rapidly with inadequate aeration. If the maximum solution rate is fairly close to the peak oxygen demand, the organism will be adequately aerated over most of the growth period and then slightly under-aerated over a relatively short period. This period, however, seems to be a critical one for antibiotic production, and considerable loss occurs if the required supply of oxygen is not then available.

It is quite likely that, with some of the simple apparatus which is frequently used for screening large numbers of strains, failure to obtain the best titre of which a strain is capable might be due to poor aeration. For instance, a 1 l. conical shake flask, containing 300 ml. of medium and oscillating 90 times per min. with a 7 cm. throw, had a maximum solution rate of 0·4. Again, a large test-tube, fitted with a wash-bottle head and aerated at 1 volume per volume per min., had a maximum solution rate of 0·3. A similar tube aerated through a sintered disk gave a maximum solution rate of 0·5, which is some improvement on the simple test-tube. Adequate aeration was obtained in this apparatus by increasing the rate of air flow. When this was done only 1 % of the oxygen in the air bubbled through the medium was being consumed by the organism. This apparatus does not compare unfavourably for efficiency of aeration with most of the culture vessels commonly used.

This waste of oxygen is not usually important on a laboratory scale, but on an industrial scale the cost of aeration may be very considerable. It is therefore important to design efficient large-scale vessels, and by suitable small-scale experiments to find out the minimum aeration which is required for economical running of the plant. Although, so far, little attention has been paid to the point, it would probably be useful to try to select strains which give a good titre with little aeration.

The technique described should be applicable to any type of submerged cultures. It is likely that most of the oxygen demands encountered would be of zero order, and in this case any given metabolic process should eventually become independent of the rate of aeration. However, the method is applicable to any culture in which the oxygen demand can be expressed as a function of the oxygen tension, and in this case k_0 in equation (2) would be replaced by the appropriate function.

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The Serology of Fifty Strains of *Proteus vulgaris*

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SUMMARY: Of fifty strains of *Proteus vulgaris* from human infections, seven of the 'vulgaris' and forty-three of the 'mirabilis' fermentative type, forty-four were brought into Belyavin's phase A, which represents the modal S form of recently-isolated strains, and analysed serologically by their H and O antigens. Three of the six remaining strains were irreversibly R, and three were too unstable antigenically for the routine analysis.

The antigens determining the somatic O types were resistant to heating at 95°, to ethanol and to dilute HCl, treatments that destroyed the H antigens. Serologically similar O antigens were extracted from the bacilli by hot formamide and by ethanol precipitation of tryptic digests.

As in other recent surveys of Proteus serology, the classification of strains by major O antigens was more clear-cut than by H antigens, though some minor O antigens were distributed differently from the major. The forty-four phase-A strains fell into eighteen O-antigenic types, three of these being the classical OX2, OX19 and OXK types, strains of which were included in the survey. The H antigens of each strain were often apparently multiple, but grouping by major H antigens was broader than by O antigens; moreover, H antigens were often common to strains with dissimilar O antigens.

Apart from the antigenic changes accompanying variation from phase A to phases B or C, there were suggestions of other phasic variations in both H and O antigens, which, if substantiated, may necessitate a reconsideration of serological classifications of the group. The three unstable strains in the collection could not be definitely assigned to any O-antigenic type.

The serological study of *Proteus vulgaris* and closely allied microbes covers a period of fifty years. The earlier work was directed, among other things, mainly to finding a species antigen by which *P. vulgaris* could be identified (Wolf, 1899; Klieneberger, 1908; Cantu, 1911; van Loghem & van Loghem-Pouw, 1912; Horowitz, 1916; Bengston, 1919; Wenner & Rettger, 1919; Taylor, 1928). As a result of these investigations, it was generally conceded that *P. vulgaris* was antigenically heterogeneous. One type of heterogeneity in *Proteus* spp., the distinction of flagellar and somatic antigens, was discovered by Weil & Felix who also discovered differences in the O antigens of the classical strains, X19, X2 (Weil & Felix, 1917; Weil, 1920, 1923; Felix, 1923). With the description of the XK strain (Fletcher & Lesslar, 1926) a third O-antigen complex was recognized.

Work on the flagellar and somatic antigens of the *Proteus* group during the past twenty years has defined the heterogeneity of the species antigens more fully (Moltke, 1927; Yacob, 1932; Varela, Zozaya & Imaz, 1944; Winkle, 1943, 1944; Rustigian & Stuart, 1945; Perch, 1948) with regard to both H and O antigens. In particular, Winkle (1944) and Perch (1948) have made a detailed receptor analysis of their strains and have numbered or lettered the determinant groups identified, thus allotting an antigenic 'formula' to each strain and group.

In the course of her investigations, Perch tested Winkle's type strains with her own sera.

Our serological investigation started in 1936, with the collection of 21 strains from human infections (see Miles, 1951) and of authentic stock strains for comparison. The serological analysis was interrupted by the 1939–45 war, during which most of the strains were maintained in stock nutrient agar stabs. A number of Proteus strains from infected burns were collected in 1943. On the resumption of serological work in 1946, many of the somatic serological characters previously found in the pre-war strains had disappeared or were difficult to elicit; and those even of the more recent burn strains were variable on retest. For this reason, the status of each stock strain was re-investigated (Belyavin, 1951), with the discovery of a modal phase A, equivalent to the fully Smooth form of other Gram-negative enterobacteria, and two other phases, B and C, of which B corresponds to a partially Rough form, with loss of the type O-antigen, saline-stability and virulence for mice. Since the variations A→B and A→C were reversible, it proved possible to coax most of the degraded stock strains into phase A, and we report below an analysis of the O and H antigens of *P. vulgaris* in Belyavin's phase A.

Symbols. We have used the sign PR, followed by a number, to designate our strains, except for those which were already named (X 2, X 19, etc.). Suspensions treated to destroy flagellar antigens, and the corresponding antisera, are labelled OPR 1, OPR 3, etc.; and suspensions of flagellated forms and antisera to them, HPR 1, HPR 3, etc. A collection of strains having the same phase A O-antigen constitute a 'type', and those with the same main H antigen, a 'group'; but we intend these terms to be only temporary descriptions pending an agreed designation for the Proteus antigens.

Character and source of the strains

The strains were isolated from human urinary, wound, pleural and burn infections (where they were either the sole or the predominant microbe as judged by examination of morbid material and direct plate cultures), with the exception of six strains from collections, and PR 37 and 39, from laboratory mice dying of an intercurrent spontaneous Proteus infection during toxicity tests with Brucella endotoxins.

All strains were typical *P. vulgaris*, swarming on 2 % nutrient agar, liquefying gelatin, producing H_2S from peptone water and splitting urea with the production of NH_3. Of the fifty strains, seven (PR 5, X 2, XL, X 19, PR 29, 37, 39) were '*P. vulgaris* Hauser' (Bergey, 1948), in that they readily fermented maltose and sucrose, and readily produced indole. One (PR 36) readily though feebly fermented maltose and sucrose, and was indole-negative; and the remaining forty-two were '*P. mirabilis* Hauser' (Bergey, 1948), being indole-negative, and failing to ferment maltose or sucrose within 5 days. Both the 'vulgaris' and 'mirabilis' varieties are called *P. vulgaris* throughout this paper.

Pus strains. PR 5, 7, 9, 10, 13, 19, 21.

Urinary strains. PR 1, 3, 4, 11, 12, 15, 16, 17, 18, 20, 22, 23, 31, 32, 33, 35, 36.

Mouse strains. PR 37 and 39.

Burn strains. PR 41–52, isolated in 1943 by Dr L. Colebrooke from infected burns in ten patients during a ward outbreak of Proteus infection; and PR 53–58, from five patients during a second outbreak in a burns ward.

Stock strains. Proteus X2; *Proteus* X19; *Proteus* XK; *Proteus* XL (all from Col. H. J. Bensted); PR 29, *P. vulgaris* originally from Prof. Rettger (Wenner & Rettger, 1919); PR 30, N.C.T.C. 3156, selected because it was reported to react serologically with Brucella antisera. We also received from Dr F. Kauffmann seventy-one of the strains used by Dr Perch in her analysis, representing forty-nine of the somatic groups described in the simplified antigenic schema of Kauffmann & Perch (Perch, 1948). Some of these strains were tested against sera representing seventeen of our somatic types, with a view to identifying receptors common to the two classifications.

Up to 1945, the strains were maintained in soft agar stabs, subcultured at 6-weekly or 2-monthly intervals; in 1946 and after, they were kept in the dry state by Stamp's (1947) method.

METHODS

Preparation of phase A strains

Stability of suspensions. The strains were tested for thermal, saline and acriflavine stability. Thick suspensions in distilled water were killed by heating for 1 hr. at 56°, and diluted to 2×10^9 cells/ml. for the saline, and to 8×10^9/ml. for the thermal tests (Pandit & Wilson, 1932), the density being estimated in a simple visual comparator against a counted suspension of 2×10^9 *Salmonella typhi*/ml. The 8×10^9/ml. suspensions were heated in a 95° bath for 60 min. and examined during the heating at 15 min. intervals; and the 2×10^9/ml. suspensions mixed with equal volumes of 5, 4, 3, 2, 1 and 0% solution of NaCl, and read after 18 hr. incubation at 50°.

The acriflavine test, described by Pampana (1933), was made on a number of single colonies on MacConkey agar cultures of each strain by suspending them in a drop of 0·85% saline on a slide, and mixing with an equal volume of 1/500 acriflavine in 0·85% saline. Agglutination of the cells, readily visible with a ×3 lens within 2 min., was recorded as positive. It is clear from Table 1 that acriflavine stability is not associated with saline and thermal stability. Heat-stable suspensions give rise as often as heat-labile suspensions to acriflavine unstable colonies and in comparable proportions; and in only one instance did the selection of an acriflavine-stable colony affect the heat stability of the strain. Comparison of the acriflavine and the saline tests is less valid because saline stability was at first tested with flagellated suspensions. Later work proved that flagellated suspensions were often saline-stable when the derived O suspensions were unstable. In any series of colonies from a strain quite stable to both heat and saline, acriflavine-stable colonies may nevertheless be exceedingly rare. We finally abandoned the acriflavine test as a method of selecting smooth strains. The decision was also justified by the fact that many variants, which later proved to be antigenically degenerate and to have lost virulence, were as acriflavine-stable as the parent strain.

Table 1. *The relation of thermal, saline and acriflavine stability of flagellated strains of* Proteus vulgaris

Strain	Source	Agglutination by Saline	Agglutination by Heat (95°)	Acriflavine test*
PR 1	(*a*) From stock	+	0	3/20
	(*b*) Acriflavine-stable, from (*a*)	0	0	6/10
	(*c*) After mouse-passage	0	0	0/5
PR 3	(*a*) From stock	0	0	2/20
	(*b*) Acriflavine-stable, from (*b*)	0	0	3/9
	(*c*) After mouse-passage	0	0	0/5
PR 23	(*a*) From stock	+	+	2/20
	(*b*) Acriflavine-stable, from (*a*)	tr.	0	2/6
	(*c*) After mouse-passage	0	0	0/5
PR 4	From stock	0	0	0/7

* Number of colonies positive/number tested.

Selection of colony types for preparation of suspensions. All the parent strains and variant forms discovered during the investigation could be assigned to one of the three phases described by Belyavin (1951). The colonial types found could be divided into a modal phase A, which always gave rise to saline-stable O suspensions, and at least two other phases of which one (B) had an antigenic constitution of different specificity when tested against a series of fifteen somatic antisera, and tended to be unstable in saline suspensions. Whereas the phase A antigens were type specific, the antigenic complex of the B variant had two new antigens, one of broader specificity, a cosmopolitan antigen, the other narrower—indeed, strain specific. These components were present on the surface in varying amounts as judged by agglutination and absorption tests, together with small amounts of the type-specific antigen; moreover, even predominantly type-specific strains contained small amounts of the B phase-antigenic complex. These antigenic components could be detected in suspensions by the use of appropriate antisera, and serological tests of suitability eventually replaced those for saline and thermal stability.

Flagellar (H) suspensions. The dry stock cultures were seeded into CCY broth (Gladstone & Fildes, 1940) incubated for 5–6 hr. at 37° and plated on to MacConkey's agar. The next day, single colonies were seeded on to nutrient agar, the 20 hr. growth washed off with distilled water, centrifuged once, and resuspended in distilled water. The suspension was killed by heating at 56° for 1 hr. and preserved by 0·25–0·4 % formalin. Such suspensions were adequate for absorbing flagellar antibody, but in many instances were inferior to liquid cultures as agglutinating suspensions. Liquid cultures were prepared by inoculating single colonies into 40 ml. of 1 % peptone water, incubating for 3–5 hr. at 37° and then at 22–25° for 3 days. The cultures were killed by heat and formalized without washing.

On the whole, slightly higher titres were obtained with the formalized peptone cultures, and the agglutination was more floccular. However, the flagellar agglutination of a strain was sometimes variable, and occasional subculture by swarming over a 2 % agar surface, or through 0·5 % nutrient agar in U tubes was necessary to produce a strongly agglutinating flagella suspension. With some strains even this treatment failed.

Somatic (O) suspensions. Since non-motile, non-swarming strains may be flagellated, though poorly (Colquhoun & Kirkpatrick, 1932) we did not attempt to induce non-motile variant strains for making O suspensions, but relied on the destruction of H antigens by ethanol, and by acid (Duncan, 1935). One volume of 0·01 N-HCl at 37° was added to an equal volume of distilled water suspension in a water-bath. After 5 min. a one-tenth volume of 0·1 N-NaOH was added to stop the reaction. The suspension was centrifuged once, resuspended in distilled water and heated for 1 hr. at 56°; preservative was not added. The rate of destruction of flagella at a given temperature and acidity is a function of bacterial density (Duncan, 1935). We confirmed that this treatment completely destroyed all flagellar antigen in suspensions containing 100×10^9 cells or less/ml.; all suspensions for acid treatment were diluted first to this concentration. The acid method is quick and simple, but suspensions of heat- and saline-agglutinable strains became much more so during acid treatment and were unsuitable for serological tests. Ethanol treatment, on the other hand, made these unstable forms more stable (Belyavin, 1951), and because we wished to analyse all forms serologically, it was adopted as a routine. Ethanolized type-specific suspensions induced good antibody formation in rabbits, and agglutinated more strongly than type-specific, acid-treated suspensions, although the latter gave slightly more consistent end-points in repeated titrations. We must emphasize that modal phase A (type-specific) strains yielded equally stable suspensions whether they were treated with acid or ethanol and gave precisely the same serological results.

The suspensions were prepared by washing off a nutrient agar culture in 96 % methylated spirit and heating for 1½ hr. at 56°. The suspension was spun down, washed twice in distilled water, finally resuspended in distilled water and stored at +5°. No preservative was added. We did not use as a routine O suspensions prepared by heating to 100° for 30 or 60 min., as recommended by Perch (1948). With phase A strains the results with ethanol, HCl and heating to 100° were identical, but phase B and phase C strains, initially unstable, were often more so by this treatment, and it was convenient to use ethanol, which was suitable for all three phases. Moreover, the results of HCl treatment were useful in detecting phase B strains (Belyavin, 1951).

Preparation of antisera. Albino rabbits, 2–3·5 kg. in weight, were immunized by the bi-weekly intravenous injection of bacterial suspension. The initial dose was 10^8 organisms and the dose was doubled at each injection, six to seven doses being given. Usually 'first-course' bleedings were used; a second course of injections sometimes induced a broadening of specificity in the resulting antisera, and 'second-course' bleedings were seldom used.

Serological methods

Agglutination tests. Twofold dilutions of antisera were mixed with an equal volume (0·3 ml.) of suspensions containing 2×10^9 cells/ml. and held at 50°. H agglutination was read at 4 and 18 hr. and O agglutination after 18 hr. The end-point of the titrations was the reciprocal of the serum dilution producing agglutination barely perceptible to the naked eye, but easily visible with a × 3 hand-lens when viewed by oblique illumination against a dark background ('standard' agglutination). For intermediate values in the two-fold series, interpolation factors were calculated by the method of Dreyer & Inman (1917). As in the Salmonellae, H and O agglutination respectively were floccular and granular.

Slide agglutinations were used in screening tests of O suspensions against type sera. Two loopsful (2 mm. loop) of neat serum were mixed with *c.* 0·05 ml. of ethanolized O suspension containing about 50×10^9 cells/ml., i.e. a final serum dilution of 1/3–1/5. The test was read in oblique transmitted light after rocking the slide for 1–2 min. The agglutinations were quite clear-cut and easy to read. Slide agglutination tests are liable to be falsely negative when compared with tube titrations, unless highly diluted sera are used. To establish the reliability of the slide test, ninety titrations were made in parallel (Table 2).

Table 2. *The relation between slide and tube agglutination tests of O suspensions of* Proteus vulgaris

Serum no.	Slide negative			Slide positive			Totals
	Tube negative	Tube positive		Tube negative	Tube positive		
		Up to 20	>20		Up to 20	>20	
1	13	0	0	0	0	2	15
2	15	0	0	0	0	0	15
3	12	1	0	0	0	2	15
4	14	1	0	0	0	0	15
5	13	2	0	0	0	0	15
6	11	0	0	0	2	2	15
Totals	78	4	0	0	2	6	90

In ninety tests, four were slide-negative but tube-positive. All four, however, gave tube titres, which, being less than $\frac{1}{100}$th part of the homologous titre, were not significant in type identification. A similar series of thirty tests with four other sera, but containing a higher proportion of slide-positive suspensions gave similar results. It was not uncommon to find that slide-positive suspensions were agglutinated only to low titres in tubes. The slide test then is reasonably reliable for 'screening' ethanolized O suspensions.

Absorption tests. Two methods of absorption were used. In one, the serum at a fixed dilution (1/10 or 1/20) was absorbed by an equal volume of falling concentrations of antigen, and after centrifugation the supernatant from each tube tested directly against one or more suspensions. In the other, after absorption of the serum with a single concentration of antigen, the residual antibody was titrated in the usual manner. In both cases the absorption was carried out for 2 hr. at 37°. Both tests were used in parallel, one acting as a check on the other, when small numbers of strains and sera were involved. When more than three strains and three sera were tested the second method was used as being more economical of time and materials. Table 3 exemplifies both methods in determining the antigenic relationship of two strains falling within the same somatic type. When a serum was absorbed, particularly by a heterologous strain, the agglutinins were usually more completely removed by low concentrations of absorbing antigen than with high, as is evident in Table 3*a*. For absorption of sera in bulk, therefore, a low multiple of the minimal absorbing dose was always used. For a large number of suspensions, the absorbed sera were first tested by the slide method to eliminate non-reacting strains.

Table 3. *'Mirror' cross-absorption tests in which a given serum dilution is (a) absorbed by graded doses of antigen, and (b) by a single dose of antigen* (5×10^9 cells/ml.) *with subsequent titration of residual antibody*

(*a*)

Serum (1/10)			Initial conc. of absorbing suspension (10^9/ml.)					
			80	40	20	10	5	0
			Agglutination result					
Against	Absorbed by	Tested against						
PR 7	OPR 7	OPR 7	S	S	tr.	tr.+	T−	T−
		OPR 10	0	0	0	0	0	T−
	OPR 10	OPR 7	T−	S+	T−	S+	T−	nt
		OPR 10	tr.	tr.	tr.	tr.+	tr.	nt
PR 10	OPR 7	OPR 7	tr.	tr.+	tr.+	tr.+	tr.+	T−
		OPR 10	T−	T−	T−	T−	T−	T−
	OPR 10	OPR 7	0	0	0	0	0	nt
		OPR 10	S	S+	S+	0	0	nt

tr., tr.+, S, S+, T−, T = degrees of trace, 'standard' and total agglutination; nt = no test; 0 = no agglutination.

(*b*)

Serum (1/10)	Tested against suspension	Titre after absorption with		
		Nil	OPR 7	OPR 10
OPR 7	OPR 7	1280	80	640
	OPR 10	320	<20	20
OPR 10	OPR 7	320	20	<20
	OPR 10	1280	640	<20

RESULTS

The phase A somatic antigens

Table 4 shows the cross-reactions of HCl-treated suspensions from strains with their corresponding sera. It is arranged to display the main agglutinating types. A few are singleton strains, that is, they react only with their own sera, and their sera do not agglutinate any other strain in the collection. A summary of all types with their component strains is given in Tables 5*a* and *b*. Eighteen serological types emerge from forty-four strains, of which ten are singletons. Three strains, PR 5, 20 and 41, were irreversibly rough when tested in 1948. It was not possible to classify PR 19, 21, and 22 by agglutination reactions, because the strains were unstable, with a variable antigenic type. Both PR 19 and PR 21 at times exhibited affinities with the X 2 type and PR 19 and PR 22

Table 4. *The O-agglutination reactions of thirty-one sera and thirty-four phase A strains of* Proteus vulgaris

O-suspension of (PR)	O-Antisera to (PR)																														
	4	9	31	3	13	15	23	XK	30	42	11	17	45	7	10	X 19	XL	X 2	44	46	47	50	52	51	53	1	12	43	32	37	39
4	1280	1000	5120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	0	0	0	0	0	0	40	0	0	40	0	0
9	1280	1280	5120	20	0	0	0	0	0	0	0	120	0	0	0	20	120	40	0	0	0	0	0	0	0	0	0	0	20	0	0
31	640	1280	5120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0	0	0	30	0	0
3	0	0	0	640	2560	1000	160	2560	0	0	0	0	0	20	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0
13	0	0	0	320	2560	2560	640	5120	0	20	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	640	1280	5120	1280	5120	0	20	0	0	0	0	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	320	1280	640	1920	10240	20	480	0	60	0	40	20	0	0	20	0	0	0	10	20	0	10	0	0	0	0	0	0
XK	20	0	0	60	240	320	640	10240	20	1000	0	20	0	40	0	20	20	0	0	0	0	0	0	0	0	60	0	20	0	0	0
30	20	20	0	20	160	320	480	640	480	40	640	1280	40	40	20	20	20	20	10	0	0	10	10	10	10	0	0	10	0	0	0
42	0	0	0	0	40	60	20	80	160	2560	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	320	0	1280	1280	1000	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	160	0	1000	1000	2560	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0	0	0	640	1000	1280	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0	20	0	0	640	1000	2000	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45	0	0	40	0	0	0	0	0	0	0	480	1000	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	160	0	0	0	0	0	0	0	5120	1280	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	40	0	0	0	0	0	0	0	1280	5120	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
X 19	20	0	0	30	0	0	20	0	0	0	0	0	0	0	0	5120	5120	60	0	0	0	0	0	0	0	30	0	0	0	0	0
XL	0	0	0	80	0	0	0	0	0	0	0	0	0	0	0	5120	5120	40	0	0	0	0	0	0	0	0	0	0	0	0	0
X 2	0	0	0	40	0	0	20	0	0	0	0	0	0	0	0	0	0	2560	0	0	0	0	0	0	0	60	0	0	0	0	0
44	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5120	5120	2560	1280	1280	0	0	0	0	0	0	0	0
46	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0
47	0	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	5120	2560	1280	1280	0	0	0	0	0	0	0	0
48	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0
50	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0
52	0	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3750	3750	2560	1280	1280	0	0	0	0	0	0	0	0
51	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	640	0	0	0	0	0	0	0
53	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1280	0	0	0	0	0	0
1	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	1280	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1280	1280	0	0	0	0
43	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	0	0
37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	3800
39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2560	2560

with type 10. The lability of these strains is well exemplified by the behaviour of PR19. The stock strain was at first Rough, but the apparently stable phase A selected from it reacted moderately with an X2 antiserum. Tested 4 days later, it had lost its X2 reactivity, and agglutinated to low titre with a type 10 serum; when tested a week later, it would react with none of the phase A sera. The other two varied analogously but both finally reached a state when they reacted to a very low titre with over half a dozen phase A sera, as though they were now characterized by a minor 'cosmopolitan' antigen. No serum was prepared against PR58, the fiftieth strain, but since it failed to agglutinate with any of the type O-antisera, it is presumably a singleton.

Table 5*a*. *The O antigenic types of forty-four phase A strains of* Proteus vulgaris, *including the four classical OX strains*

Somatic type	PR strains	Total
1	1, 12	2
2	4, 9, 31	3
3	11, 17, 18*, (30), 35, 45, 49, 56	8
4	7, 10	2
5	44, 46, 47, 48, 50, 52, 54, 55, 57	9
6	51	1
7	53	1
8	29	1
9	43	1
10	32	1
11	36	1
12	33	1
13	37, 39	2
14	16	1
15	58	1
X 2	X 2	1
X 19	X19, XL	2
XK*a*	23, (42), XK	6
b	3, 13, 15	
	Total	44

( ) = Strains carrying additional type antigens.
* See text (p. 187).

Table 5*b*. *H antigenic groups of twenty phase A strains of* Proteus vulgaris, *including the four classical X strains*

Flagellar group	PR strains	Total
A	4, 9	2
B	1, 3, 10, 12, 13, 15, 23	7
C	7, 11, 17, 19, 22, X 2, X 19, XK, XL	9
D	16	1
E	21	1

In both tables, numbers in **heavy type** indicate strains against which sera were prepared.

Types containing more than one strain are discussed below, especially those that are heterogeneous with respect to their minor antigens.

Somatic types 1, 2 *and* 3. These three types, containing two, three and eight strains respectively, are each antigenically homogeneous; each component strain will remove all the O antibody from the type sera. This, of course, does not preclude the possibility of strains belonging to these types, with heterogeneous minor antigens. Strain PR18 is not classed as type 3 by agglutination or absorption tests; the strain is rough, although strain-specific receptors are present. However, it contains a trace of the type 3 antigen, because it induces type 3 antibody in the rabbit.

Type XK. This type certainly includes five strains: PR3, 13, 15, 23 and XK and possibly PR42. Strains PR3, 13 and 15 agglutinate to a lower titre than strains PR23, and XK with OXK antiserum. Conversely, strains XK and PR23 agglutinated to low titres with PR3 and 15 antisera. The cross-adsorption tests with ethanolized suspensions (Table 6) subdivide the type into two, *a* and *b*, the classical XK strain falling in type XK*a*. All these strains are antigenically distinct from the other X strains, X2, X19 and XL.

Table 6. *Cross-absorption of tests of type OXK strains*

		Titre after absorption by					
Serum	Tested against suspension	Nil	OPR 3	OPR 13	OPR 15	OPR 23	OXK
OPR 3	OPR 3	320	0	0	80	160	160
	OPR 13	160	0	0	40	80	80
	OPR 15	80	0	0	<20	<20	<20
	OPR 23	40	nt	nt	nt	nt	nt
	OXK	40	nt	nt	nt	nt	nt
OPR 15	OPR 3	320	0	0	0	80	80
	OPR 13	320	0	0	0	0	0
	OPR 15	1280	0	0	0	0	0
	OPR 23	100	0	0	0	0	0
	OXK	80	0	0	0	0	0
OXK	OPR 3	640	160	120	40	0	0
	OPR 13	1280	240	160	80	0	0
	OPR 15	1280	240	240	160	0	0
	OPR 23	2560	480	480	480	0	0
	OXK	2560	1280	1280	1280	0	0

nt = no test because unabsorbed serum too weak.

Difficulties in classification are raised by the behaviour of strains PR30 and PR42. PR30 reacts strongly with sera for subtypes *a* and *b*, but PR42 does not react with the subtype *a* sera, and only agglutinates to 5 % of full titre with subtype *b*. Nevertheless, its own serum agglutinates OXK to at least 50 % of full titre; moreover, PR30 carries an antigen reacting to full titre with the sera of the otherwise quite distinct type 3, and PR42 has an antigen not related to any other in the collection. It is not clear, therefore, whether these strains should, in fact, be referred to the XK type. Two of Dr Perch's strains behaved similarly, i.e. as though possessing an XK antigen in addition to that characterizing a different somatic type. We have no evidence that such an antigenic constitution is quantitatively variable, with one or other type antigen predominating at the surface in different phases. If phase variation of this kind were proved, the validity of the XK type antigen as a constant feature characterizing XK strains would need reconsideration.

Type 4. This consists of two strains, PR 7 and PR 10. They cross-agglutinated, but not to reciprocally equal titres. The two strains are not antigenically homogeneous (Table 3*a*).

Type 13. This consists of two antigenically homogeneous strains, PR 37 and 39.

Type X 2 *and X* 19. These types, although antigenically distinct, may be considered together, as they are represented in the collection by only three strains, viz. X 2, X 19, and XL. Strain X 2 is a singleton. On the other hand strains X 19 and XL as originally described are closely related although not homogeneous (Table 7). Whereas XL removes agglutinins for both itself and X 19 from anti-XL serum, X 19 even in high concentration, removes only its own antibody. Conversely, XL does not remove all antibody from the X 19 antiserum, although it obviously removes more of its own agglutinin. The relationship of these two strains is of considerable interest. The results described suggest that they differ in their somatic antigens only by the presence of an additional receptor in strain XL, and in this we agree with Kauffmann & Perch (1947). On the other hand, Winkle (1944) allots the additional antigen to strain X 19 and identifies it also in strain X 2. The ethanolized OXL suspension used gave a well-marked though partial agglutination to a titre of 640 with a strong phase B serum (58α) which did not occur with OX 19. The antigen that distinguishes XL from X 19 may therefore be a phase B antigen. The two strains may be even more complex. A bacteriophage from the Pasteur Institute reported to act on the Proteus strain OXL, when tested against subcultures of X strains obtained from the National Collection of Type Cultures, indeed attacked the strain XL but not X 19. In the case of the X strains we received from Col. Bensted, however, OX 19 was attacked and not OXL. This discrepancy is unexplained.

Table 7. *Cross-absorption tests of Proteus OX* 19 *and OXL*

Serum (1/20)			Initial conc. of absorbing suspension (10^9/ml.)					
Against	Absorbed by	Tested against	80	40	20	10	5	0
OXL	OX 19	OX 19	0	0	0	S−	T	T
		OXL	S+	T−	T−	T	T	T
	OXL	OX 19	0	0	0	0	S	nt
		OXL	0	0	0	S+	T−	nt
OX 19	OX 19	OX 19	0	0	0	T−	T	T
		OXL	0	0	0	T−	T	T
	OXL	OX 19	S+	S+	tr. +	T−	T−	nt
		OXL	S+	S+	tr.	tr. +	T	nt

Symbols as in Table 3.

Types 6, 7, 8, 9, 10, 11, 12, 14 *and* 15. These types are all represented by single strains. It is possible that some of these, notably PR 16, are in a 'strain-specific' phase. Type 9 contains PR 36. This strain has a 'rough' colonial morphology and tends to be unstable in high concentrations of saline. The creation of an O type to accommodate this strain must be regarded as tentative.

Somatic antigen-analysis by precipitin reaction. Two kinds of soluble extract were tested against O-antisera, and gave identical results. The first was obtained by ethanol precipitation of tryptic digests of acetone-extracted cells (Miles, 1951) and the second, more simply by treating the centrifuged deposit from 2 ml. of a 5 or 20 hr. culture in CCY broth with hot formamide, by the method of Fuller (1938). The extract was layered on undiluted serum and precipitation at the interface occurred within 10 min. at room temperature. Eighteen strains were tested against sixteen sera (Table 8). The reactions confirm the results with cross-agglutination tests (Table 4), but strains belonging to antigenically heterogeneous types are sharply separated into subtypes without cross-precipitation; e.g. the reactions between PR3, 13, 15, 23 and XK, and of PR7 and 10. The effect might be due to selective extraction of different fractions, or to a concentration effect; the same sera were used for agglutination and precipitation, and, being induced by one or at most two courses of injections, perhaps contained insufficient antibody for a visible heterologous precipitin reaction. The formamide method which can be applied to 2 ml. of a 5 hr. culture would clearly be useful for rapid O typing of Proteus strains.

Table 8. *The cross-precipitation reactions of sixteen antisera and formamide extracts of eighteen phase A strains of* Proteus vulgaris

	O-Antisera to (PR)															
Extracts from (PR)	4	9	3	23	XK	30	11	17	7	10	X19	XL	X2	1	21	32
4	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
13	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
23	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—
XK	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—
30	—	—	—	—	—	±	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—
17	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—
X19	—	—	—	—	—	—	—	—	—	—	+	±	—	—	—	—
XL	—	—	—	—	—	—	—	—	—	—	±	+	—	—	—	—
X2	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—
32	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+

The flagellar antigens

Table 9 shows the flagellar cross-agglutination titres for nineteen of the strains in the collection; the analysis was not carried beyond this point. Eighteen fall into five groups, containing respectively one, one, two, seven and nine strains and comprising strains belonging to nine O types. The word 'group' is not used here in the strict sense of a serological class, but merely implies a collection of strains, and these we designate A, B, C, D and E (Table 5*b*).

They are not as clearly defined as the O types by the cross-agglutination reactions, and cross-absorption tests reveal that all are antigenically heterogeneous.

Table 9. *The H-agglutination reactions of fifteen sera and nineteen phase A strains of* Proteus vulgaris

H-suspension of (HPR)	H-antisera to (HPR)														
	9	4	1	3	13	23	15	XK	19	17	11	X19	X2	XL	16
9	2560	7600	60	0	0	0	0	0	0	0	80	0	80	0	0
4	3800	10240	60	0	0	0	0	0	0	0	80	0	160	0	0
12	0	0	1280	5120	0	120	0	0	0	0	0	0	0	0	0
1	0	0	1280	5120	3800	>10240	30	2560	0	0	0	0	0	0	0
3	0	0	2000	20000	250	160	3800	640	0	0	0	0	0	0	0
13	320	0	480	1280	5120	2560	480	480	0	0	0	0	80	0	0
23	0	0	1000	120	1280	>20480	640	15360	0	0	0	0	60	0	0
15	320	160	1280	7600	320	80	5120	120	0	0	0	0	80	0	0
10	0	0	750	5000	3000	160	<40	10000	5000	5120	160	nt	0	0	0
7	0	0	0	0	160	0	80	15360	160	160	3800	3800	nt	750	0
XK	0	0	0	160	0	160	40	15360	240	2560	5120	2560	1280	640	0
19	0	0	0	0	0	0	0	640	3800	3800	0	0	0	0	0
17	0	0	0	0	0	0	0	160	nt	5120	80	<40	<40	<40	0
X19	0	0	0	0	0	0	0	20480	160	640	1280	5120	1280	1280	0
X2	0	0	0	160	0	0	0	10240	120	5120	3800	2600	1280	1000	0
XL	0	0	0	0	0	0	0	15360	1000	5120	3800	1280	1280	1280	0
11	0	0	0	60	0	0	0	15360	0	1280	5120	2560	2000	1280	0
22	0	0	0	0	0	0	0	15360	0	160	1280	3750	0	160	0
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7620

nt = no test.

A, D and E groups. PR4 and 9 comprise the A group. Their flagellar antigens are not entirely homogeneous, and they differ in at least one receptor. D group is represented by PR16, and E by PR21. Both have an antigenic flagellar constitution distinct from that of the other strains examined.

B group. Strains PR12, 1, 3, 13, 23, 15 and 10 constitute B group. Cross-absorption of three anti-sera HPR3, HPR23 and HXK by four of the strains (Table 10) show at least three distinct antigens and from the cross-agglutination tests of PR12 and XK, two others may be inferred. One receptor carried by HXK is common to the whole group.

C group. This is formed by the strains PR7, PR19, PR17, X19, X2, XL, PR11 and PR22. XK can also be considered as falling mainly in this group, but its exact relationship is discussed below. The cross-absorptions with three of the main sera (Table 11) reveal the distribution of four antigens. The strains 19 and 17, as they agglutinate only with their own sera and with that of XK, can be considered as forming a minor subdivision and might with justification be taken out of the major group of strains. They are retained, however, on the basis of their antigenic relationship with strain XK.

The flagellar antigens of PR7, 11, 22 and strains X19, X2, XL and XK have something in common. It is noteworthy that XK has wide antigenic relationships, and it is of particular interest that although the antiserum against XK agglutinates both group B (PR1, 3, 12, 13, 15, 23, 10) and group C (PR7, 19, 11, 22 and strains X19, X2, XL) to a high titre, it was itself agglutinated only by the sera of group C (Table 9).

Table 10. *Cross-absorption tests of strains in flagellar antigen, group B*

		Titre after absorption by				
			(HPR)			
Serum	Tested against suspension (HPR)	Nil	1	3	23	XK
HPR 3	1	5120	<80	<80	80	320
	3	20000	5120	1920	5120	3800
	13	1280	<80	<80	<80	<80
	15	7600	5120	1280	5120	5120
	23	120	<80	<80	<80	<80
HPR 23	1	>10240	80	5120	80	3800
	3	160	<80	<80	<80	<80
	13	2560	<80	2560	<80	2560
	15	80	nt	nt	nt	nt
	23	>20480	80	1920	<80	2560
HXK	1	2560	<80	<80	<80	<80
	3	640	<80	<80	<80	<80
	13	480	<80	<80	<80	<80
	15	120	<80	<80	<80	<80
	23	15360	<80	<80	<80	<80

Table 11. *Cross-absorption tests of strains in flagellar antigen, group C*

		Titre after absorption by				
			(H)			
Serum	Tested against H suspension	Nil	XL	X2	X19	XK
HXK	XK	15360	10240	10240	10240	80
	PR19	640	<80	<80	<80	<80
	PR17	160	<80	<80	<80	<80
	PR11	15360	160	80	160	5120
	XL	15360	2000	1000	160	10240
	X2	10240	2000	160	160	10240
	X19	20480	2000	160	640	15360
HX19	XK	2560	<80	<80	<80	<80
	PR19	<40	nt	nt	nt	nt
	PR17	<40	nt	nt	nt	nt
	PR11	2560	<80	<80	<80	<80
	XL	1280	<80	<80	<80	640
	X2	2600	<80	<80	<80	1280
	X19	5120	<80	<80	<80	1000
HPR11	XK	5120	<80	<80	<80	<80
	PR19	<40	nt	nt	nt	nt
	PR17	80	nt	nt	nt	nt
	PR11	5120	<80	<80	<80	<80
	XL	3800	<80	<80	<80	<80
	X2	3800	<80	<80	<80	160
	X19	1280	<80	<80	<80	160

nt = no test, because unabsorbed serum too weak.

The anomaly may have its basis in a diphasic variation. We attempted to discover such a variation by absorbing two portions of the XK antiserum with HX 19 and HXK respectively, and diluting the absorbed sera so that they were monospecific for HXK and HX 19 respectively. The monospecific titres were about 1/30. Single colonies on MacConkey agar plated with the N.C.T.C. HXK and with Col. Bensted's strain of HXK were tested against both sera by the slide method. All colonies from the first strain were agglutinated equally well by both sera, whereas those from the Bensted strain went feebly or not at all with the monospecific 'HXK' serum, and in very different degrees with the monospecific 'HX19' serum. The matter was not pursued, but the results, though providing no evidence for diphasic variation, suggest that the content, or the development of the flagellar antigenic components in the same strain, may be variable. Further work on these lines is necessary.

Comparison of the antigenic complexes with those in Dr Perch's strains

Forty-four of our strains were allotted to eighteen somatic types, of which three are the classical X types, X2, X19 and XK. We examined thirty-eight of Dr Perch's seventy-one strains, representing the thirty O types fully described by Dr Perch in her extensive classification of 388 strains. We did not investigate the remaining nineteen types recorded in the simplified scheme of Kaufmann & Perch (Perch, 1948) since the details of the minor antigen are not recorded. Excluding the necessary identities of the X strains common to both series, the correspondences were:

Perch		Present paper
'O' group 30	=	'O' type 2
group 23	=	type 4
group 4	=	type 10
group 10	=	type 3

In addition, the strain F10 (Perch O group 29) agglutinated with our O type 2 antiserum to approximately 10 % of full titre, and U509 (Perch O group 28) to nearly 50 % of full titre against our OPR42 serum. Since U509 did not react with type XK sera it is provisionally assumed to share a minor antigen with strain PR42.

It cannot be asserted that the antigenic constitutions of the identified types are identical in every case, but they are clearly closely related. The lack of correspondence between the type composition of the two samples of organisms is of some interest. Perch was able to identify every one of Winkle's eight somatic types (excluding the X types) with one of her own (Perch, 1948). Some of the organisms in the two collections were isolated from geographically continuous regions, i.e. Denmark and Germany, although some of Winkle's strains came from Poland or the Polish-German frontier region. Our strains were collected in Britain (London and Birmingham). In all these collections the strains were of human origin. Perch's O group 10 (our O type 3) is particularly interesting. The strains of this group form 14·4 % of the 388 strains represented in Perch's extended scheme, and 18·2 % of the typable strains in our collection; that is, in both cases, the type is one of the most common, if not the most common, somatic antigen. It is completely absent from Winkle's strains; and since these numbered over 1000 it seems unlikely that its absence from his collection is due merely to errors of random sampling. It is conceivable that the differences in the distribution of O antigens in these collections is due to the limited geographical distribution of the various O antigenic types. Alternatively, the apparent dissimilarity in somatic antigen constitution between our collection of strains and that of Perch, may in our own series be the result of the selection only of strains that were in phase A or could be induced to revert to it, with the consequent elimination of certain antigens found widely distributed in degenerate strains which are characterized by the more cosmopolitan phase B and C antigens.

Applications of Proteus typing

Much information of epidemiological and clinical interest may emerge from typing Proteus strains. In this connexion it is worth recording the O types of strains PR 41–58 isolated on two occasions from infected patients in a burns ward (Table 12). The result, though not conclusive, suggests that the hospital infection of wounds and burns, recognized with staphylococci and streptococci, might also be demonstrated unequivocally with typed *P. vulgaris*.

On the first occasion, type 5 was found in six of ten infected patients; and patients nos. I and IV swabbed at an interval of a few days had either a mixed infection or a replacement of type by another (untypable → XK, 3 → 5). On the second occasion, investigated a few weeks later, types 5 and 3 are still prevalent.

Table 12. *O-antigenic types isolated during two outbreaks of Proteus infection in burns*

Patient	Type of *P. vulgaris*	Patient	Type of *P. vulgaris*
I	(not typable) XK	VIII	5
II	9	IX	6
III	5	X	5
IV	3 5	XI	7
V	5	XII	5 5
VI	5	XIII	3
VII	3	XIV	5
		XV	15

The strains isolated from patients I, IV and XII were in each case obtained within a few days.

DISCUSSION

Our analysis of the fifty strains is incomplete in two senses: (*a*) we have not made antisera to all the H strains, and so do not know the entire cross-reactivity of the collection; and (*b*) we have based our classification on the orthodox O and H antigens. With regard to (*a*), we felt it sufficient to demonstrate, as others have recently done, the antigenic heterogeneity of the *P. vulgaris*; the fact that H antigens are shared by strains with quite different O antigens; the occurrence of OX strains (in our limited collection only type OXK) in situations remote from typhus infections; and a concordance of some of our types with those in other collections. We do not wish necessarily to perpetuate our numbered typing of O antigens, nor the lettered grouping of H antigens. The proper naming of the types and groups must follow concerted action by workers in the field, whose strains are extant.

With regard to (*b*), our O antigens are those which, as bacterial suspensions, withstand heating at 95°, and treatment with ethanol or dilute mineral acid; and which, as bacterial extracts, withstood tryptic digestion followed by ethanol precipitation or exposure to formamide at 150°. Their serology was similar, whichever of the five treatments was used. But it is quite possible that other important surface antigens are destroyed by these processes and do not appear in the analysis. Moreover, the analysis was made only upon strains in what we regard as the modal S form of recently isolated strains—the phase A of Belyavin—and it is possible, by extending the analysis to antigens that predominate on the surface of phase B and phase C strains, that significant grouping might appear. The existence of phasic changes is suggested both by our own work and by Perch (1948) who described the induction of new flagellar antigens in three strains, two of them with identical somatic antigens. She reported also 'incomplete antigens' which appeared erratically in successive cultures of a given strain, but does not appear to use them in her antigenic scheme. In any event, the serological qualities of the various phases, of which only three are yet known, necessitate a careful serological study of the antigenic configuration of common variants of *P. vulgaris*, including its Rough forms, as a preliminary to a definitive classification.

Some interest attaches to the precise status to be accorded to the somatic XK type. Antigens of this type were found in two strains in our collection (PR 30 and PR 42) which also carried antigens (type 3) of other types. Two such strains (F 58 and F 151) were also detected in the seventy-one strains received from Dr Kauffmann. Winkle (1944), in his somatic antigenic table, lists two other somatic groups which carry one antigen common to the OXK group, in addition to antigens characteristic of their own. Hemmes (1948), in a survey of ninety strains of *P. vulgaris*, tested serologically against eight scrub-typhus sera agglutinating the OXK strain to a high titre, reports the wide distribution of XK antigen, which she identified in a very large proportion of her strains both by agglutination and absorption tests. Only five rabbit anti-proteus sera were used. It is noteworthy that thirty-four of her ninety strains were autoagglutinable.

The sharing of antigens from groups other than OXK is not found in any strain in our series, which however is limited in number. In the 388 strains analysed in the extended schema of Perch, however, only one other strain is listed as sharing a somatic antigen from a group other than OXK.

Winkle gives three somatic groups with one antigen in common, distinct from any found in the OXK group. According to Perch, however, these three somatic groups form three subgroups of her own somatic group 23, and may therefore merely represent the members of a heterogeneous somatic group. The evidence therefore points to the XK group as being the outstanding possessor of this peculiarity. The question therefore arises as to whether some of the OXK antigens concerned are not characteristic of a distinct phase in an antigenic variation.

With regard to the relation of the 'mirabilis' and 'vulgaris' varieties, it is to be noted that there we found no O antigens shared by strains of the two types; but the number of 'vulgaris' varieties in our collection was too small to give this observation much weight in determining whether the two fermentative types deserve specific rank.

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Cultural and Serological Phases of *Proteus vulgaris*

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SUMMARY: Three phases of *Proteus vulgaris*, A, B and C, are distinguishable by cellular and colonial morphology, and to some extent serologically. Phase A is the modal form of freshly-isolated strains; it has a uniform bacillary morphology, swarms intermittently on nutrient agar, and forms stable suspensions in 0·85 % saline. Phase B strains, though possessing flagella, are usually non-motile, non-swarming, and highly pleomorphic; usually form unstable suspensions. Phase C strains are uniformly filamentous, motile, often swarm in a continuous film, and stability in saline varies with the strain.

The somatic surface of phase A strains is characterized by a dominant type-specific antigen, and traces of a non-specific and a possibly strain-specific antigen. In phase B strains the type-specific antigen is largely lost, and the other two antigens dominate the surface. The antigenic surface of phase C strains does not differ markedly from that of phase A.

The phase variations A→B and A→C are reversible. Phase B resembles a partially R form, in which the mouse-virulence is significantly less than that of the parent phase A strain.

The first serological study of colonial variation in *Proteus vulgaris* is that of Weil & Felix (1916, 1917) in their classical paper describing the O and H variants. This was amplified later by Weil (1920, 1923) and Felix (1923) who described variations of colonial morphology in the O strains of *P. vulgaris*. Both authors described a modal colony, and one which was opaque grew more slowly than the modal on nutrient agar at room temperature, and gave a finely granular growth in broth. In addition, Felix described a non-swarming (O) colony which had an irregular or ill-defined edge, and was granular ('griessig'). Although the small-colony variant and the apparently 'normal' type of colony to which it occasionally reverted, differed serologically from the parent 'normal' strain, no association was demonstrated between changes in antigenic constitution and colonial form. Both authors regarded this variation as a specific kind of group transformation, largely because Weil found that his small-colony variant of an X19 strain had developed an antigenic receptor identical with that found in his X2 strains. Felix confirmed this, and regarded many of his variant strains as intermediate stages ('übergangsformen') in this antigenic reconstruction.

Few of the later workers on the morphology and serology of the Proteus group mention colonial or antigenic variation (Taylor, 1928; Moltke, 1927; Varela, Zozaya & Imaz, 1944; Rustigian & Stuart, 1945; Perch, 1948), with the exception of Yacob (1932) who redescribed the O and H variants of Weil & Felix. Rauss (1936), in making an extensive investigation of the *P. morganii* group, pointed out its morphological and antigenic relationships with *P. vulgaris*. He described a granular colony with a rough surface and irregular edge, which formed an auto-agglutinable suspension in 0·85 % saline and which he regarded as Rough; but he did not examine its antigenic constitution. In view of the fact that little systematic study of variation in *P. vulgaris* appears to have been reported, the following observations are recorded.

While making a serological analysis of fifty laboratory strains of *P. vulgaris* originally isolated from infections of the urinary tract and of wounds and burns (Belyavin, Miles & Miles, 1951), three types of colony were frequently found in cultures on MacConkey's agar, which for descriptive purposes have been designated A, B and C. In this paper *P. vulgaris* indicates both '*P. vulgaris* Hauser' and '*P. mirabilis* Hauser'.

Morphology of the A, B and C forms

Colonial morphology. The colonies were studied on MacConkey's (1905) agar modified by the addition of 0·5 % NaCl.

Colony A. The A colony conforms to the text-book description of *P. vulgaris*. After overnight incubation at 37° it is 3–4 mm. in diameter, colourless, lenticular with either an entire or a finely, radially striated edge and a 'beaten copper' surface. By strong oblique transmitted light, the colony usually has a white brilliance, and under a hand-lens has a laminated structure in the form of concentric rings (Pl. 1, figs. 1, 2). The bacilli in this colony always form heat-stable, saline-stable suspensions whose antigenic consitution is classed as type-specific by Belyavin *et al.* (1951); and may be regarded as the modal S form.

Colony B. By contrast with A, the B colony is smaller, with a smooth edge, a denser centre due to its conical elevation, and a smooth shiny surface (Pl. 1, fig. 2). By oblique transmitted light it is much more homogeneous than the A colony, is only finely granular and by oblique transmitted light has a dull, dusky appearance simulating the appearance of ground glass (Pl. 1, fig. 2).

Colony C. The C colony always differs strikingly from A and B. Often larger than the A colony, and flat, its surface is rough, the edge highly irregular, usually dentate or even fimbriate. By oblique transmitted light on MacConkey's agar it is much less refractile than colony A, and is markedly granular (Pl. 1, fig. 1).

The colony of the C form is always readily distinguishable from that of A and B. It is not always easy, however, to distinguish A and B, which differ most obviously when examined by oblique transmitted light. By direct transmitted light, or diffuse daylight, except for the difference in size, in a mixture of A and B, B colonies may be easily overlooked. Furthermore, the distinctive morphological characters of the two colony types are affected by the quality of the medium and by the strain. Distinction is often difficult on excessively dry plates and on media with lower concentrations of sodium chloride. Different brands of peptone influence the colony appearance, some enhancing the brilliance of the scattered light, and some the concentric laminae of the A colony. The internal structure and the relative size of the colonies are the two characters, in that order, which are the most constant.

Cellular morphology. The cellular morphology of the three forms is clear cut on whatever medium they are grown. From MacConkey's agar, the A form consists of a profusion of regular bacillary forms (Pl. 1, fig. 3) about $0{\cdot}5 \times 5$–$6\,\mu$., in marked contrast to the morphology on the same medium of forms B and C. B is highly pleomorphic; filaments, cocco-bacillary forms of varying sizes and curiously misshapen giant forms occur in profusion (Pl. 1, fig. 4), whereas form C grows in long tangled filaments, 20–40 μ. or more in length (Pl. 1, fig. 5). Occasionally fusiform or club-like swellings are present on the filaments. The motility and swarming of these forms and their growth in liquid media are also distinctive, though deviations from the modal characters are not uncommon.

On nutrient agar overnight at 37° the A form swarms intermittently in the characteristic step-like pattern across an agar surface, and the growth consists of both short and bacillary forms. The B form does not usually swarm, although colonies of the kind described by Weil & Felix (1917) as 'tröpfchen' are occasionally seen; these are colonies surrounded by a halo of minute droplet colonies scattered over thin, filmy outgrowths. The C form swarms actively on nutrient agar and frequently gives rise to a uniform film of growth without step formation.

The swarming characters are paralleled by the motility of these organisms in liquid media. Motile strains of *P. vulgaris* are more actively motile in liquid media during the first 8 hr. incubation at 37° than after 18–20 hr., when often only a few sluggishly moving organisms can be seen. In 5–6 hr. cultures at 37° in 2 % peptone water, the A form shows many actively motile bacillary and cocco-bacillary forms, the C form many motile filaments and the B form predominantly short strings of coccoid forms which are non-motile and remain so after as much as 20 hr. incubation. However, in 5–6 hr. the B forms may spread on nutrient agar, and exhibit motility in young broth cultures. At this early phase the liquid cultures of B forms are granular with a deposit, but after overnight incubation nearly always become uniformly turbid and indistinguishable from the broth cultures of A and C forms, except that the latter show a strong, silky shimmer on shaking.

Biochemical characters. Where possible the biochemical activities of the B and C forms were compared with those of the parent A strain. Fermentation was tested in glucose, lactose, maltose, mannitol, sucrose, salicin, galactose, fructose, dulcitol, xylose, raffinose, arabinose, rhamnose, dextrin and inositol. Tests were also made for production of H_2S, liquefaction of gelatin, reduction of nitrate to nitrite and of methylene blue, indole formation, Voges-Proskauer and methyl red tests. The A and B forms of five strains, and the A and C forms of ten strains were tested, and in all cases the biochemical reactions of the B and C forms were identical with those of the parent A forms.

Saline stability of suspensions of the A, B and C forms

The saline stability of the three variants was tested by the method of Pandit & Wilson (1932). In a number of cases A, B and C colonies were isolated from the same culture and were ideal for comparison. Otherwise, wherever possible, B or C forms were tested in parallel with the parent A strain. From fifteen stock strains twenty-four strains, variant and modal, were isolated.

The variants were grown on nutrient agar overnight at 37°, the growth suspended in distilled water and divided into three parts; one left untreated, one treated for 5 min. with 0·005 N-HCl at 37° (Duncan, 1935) and one centrifuged, resuspended in 96 % ethanol and heated for 90 min. at 56°. Both these manipulations destroyed any flagellar components present. All three suspensions were centrifuged and the deposits resuspended in distilled water in a concentration of 4×10^9 cells/ml. They were tested in 0·3 ml. volumes against equal volumes of graded solutions of NaCl, giving final concentrations of NaCl ranging from 6·3 to 0·15 %, and the results read after 18 hr. at 50°. All untreated and ethanol-treated A forms were stable over the whole range; in one A form, HCl treatment made suspensions unstable in distilled water and 0·15–0·65 % of saline. The C forms varied in stability; of eight tested, four were unstable in all concentrations of saline from 0 to 6·3 % in the untreated state, and of these four, two were rendered relatively stable over the whole range by treatment either with HCl or ethanol. One of the remaining four strains became unstable after treatment with HCl and the other three were stable under all conditions. The corresponding A forms that were available were fully stable.

Of eight B forms, five were markedly unstable above a saline concentration of 0·3 %, all but two being unstable both before and after treatment with HCl. The instability was however completely abolished in every case by heating in ethanol. This phenomenon is probably similar to that observed by White in R strains of Salmonellae and ascribed by him to the presence of an ethanol-soluble Q antigen (White, 1927). That some B forms were stable before treatment with HCl and unstable afterwards may have been due to the stabilizing action of hydrophilic flagellar material, which is destroyed by the mineral acid. All forms were slightly unstable in distilled water and in saline concentrations up to 0·85 %, the instability being unaffected by ethanol treatment. The reason for this is not known.

The agglutination of B suspensions was granular, whereas the agglutinations of both treated and untreated C suspensions was coarsely floccular. The auto-agglutinating tendency of C suspensions seemed to vary directly with the length and number of filaments therein, but no systematic observations were made.

Antigenic characters

Two sera were prepared in rabbits by immunization with HCl-treated suspensions of the B forms of PR18 and X19; the titres were > 640. Ethanol-treated, stable suspensions of all the variants examined in the saline stability tests were tested against them. All the B suspensions were agglutinated by both sera, five of them to full titre. Neither A nor C suspensions showed any agglutination except one A form which was agglutinated at 1/40 by one serum. The ethanol-treated suspensions of two forms, however, showed a fine agglutination in all tubes, visible with a hand-lens, including the controls. This was probably due to heat instability, because it was observed in tests with other antisera.

Clearly the B forms differed in some antigenic component from A and C forms. Four C forms were tested by agglutination and absorption tests against homologous anti-C type and anti-A type sera, using HCl-treated, ethanol-treated and untreated suspensions. The results suggested that these did not differ markedly from their parent A strain in either flagellar or somatic receptors. It was noted that O suspensions of C variants, when tested by 'pure' absorbed O sera, still gave a coarse flocculant type of agglutination, appearing within 1 hr. at 50°. These investigations were not extensive; for instance, only two anti-C variant antisera were prepared and only the four strains tested. The serological analysis of the C variant must be regarded as preliminary.

Virulence

The B forms had some of the characters of 'rough' strains—i.e. instability in saline suspension, pleomorphism and some change of serological specificity (Arkwright, 1921). The comparative virulence of B and A strains was of interest. Five of the stock strains yielded both A and B forms, and these five pairs were used for direct comparison of virulence. The strains were subcultured from dry stock and plated, A and B colonies selected and seeded with a straight wire into serum broth. After 5–6 hr. incubation, the cultures were centrifuged and resuspended in fresh serum broth to give a concentration of about 100×10^6 cells/ml. Five graded doses were injected intraperitoneally into each of eight mice weighing 19–21 g. Young broth cultures in the logarithmic growth phase were used so that the viable count was a good estimate of the number of organisms injected. Viable counts were made on MacConkey agar by the method of Miles & Misra (1938). Regression lines were fitted to the dose-response curves obtained, and the LD 50 ratios, and their appropriate standard deviations calculated (Table 1). All the B variants were significantly less virulent than the parent A strain.

Table 1. *The* LD 50 *of A and B forms of five strains of* Proteus vulgaris

Strain	Form	LD 50 (millions)	Ratio of LD 50 B/A	Fiducial limits of error (P=0·05)
PR 49	A	43·4	—	—
	B	>176·0		
PR 50	A	71·4	0·639	0·41 –0·859
	B	110·2		
PR 51	A	95·8	0·554	0·290–0·795
	B	159·0		
PR 52	A	101·6	0·397	0·0 –0·697
	B	284·1		
PR 53	A	193·8	0·619	0·343–0·857
	B	321·3		

The serology of the B forms

Although the B forms generally proved to be non-motile and non-swarming on nutrient agar, electron micrographs of a young broth culture of a completely non-motile strain showed flagella. Ethanol-treated suspensions were therefore adopted for the study of the somatic receptors of these variants, particularly as such suspensions were completely stable in saline. Since most of the variants were appreciably auto-agglutinable even in distilled water, serological confirmation of the presence of flagellar antigen and its receptor structure was not conclusive. A modification of technique may be necessary to accomplish this.

The somatic receptors were more successfully studied. Rabbits were immunized against the five B variants used in the virulence tests. After isolation, these variants were successively plated five times on MacConkey's agar, during which the colonial morphology remained unchanged. The bacilli were washed off 18 hr. agar slopes with 96 % ethanol, heated for 90 min. at 56° and then washed by three centrifugations. They were finally resuspended in distilled water. The approximate concentration of organisms was determined by comparison of opacity with a standard suspension of boiled *P. vulgaris* in an optical comparator. For agglutinations all suspensions were made up in 0·85 % saline to a concentration of 2×10^9 cells/ml.

Freshly prepared A and B suspensions were agglutinated by their homologous sera to nearly the same titre. But whereas each serum (with one exception) agglutinated its homologous A strain and no other, the B strains all showed a marked degree of cross-agglutination (Table 2). The cross-agglutination of the A strains of PR 50 and PR 52 was due to the fact that they both belonged to the same somatic type (Belyavin *et al.*, 1951). It is reasonable to suppose that the sera contained two antibodies, specific for two distinct receptors, one type-specific and characterizing each A strain, and the other predominating in the B variants and having a wide range of cross-reactivity. Obviously both receptors were present to some extent in the B variant, because the sera contained both antibodies. To test this hypothesis further, each serum was absorbed by its homologous A and B variant; 1 ml. amounts of serum, about 64 times as concentrated as the end-point dilution, were absorbed with 1 ml. of decreasing concentration of the two suspensions for 2 hr. at 37°. The mixtures were then centrifuged, and the supernatant from each tube tested against both variants (Table 3). The results confirm the hypothesis. All A antibody was removed from the sera by low concentrations of A suspensions, but even the highest concentration (160×10^9/ml.) failed to remove the B antibody completely. On the other hand, B suspensions removed not only B antibody but also A antibody, confirming the presence of both antigens in several of the B forms. In order to examine the cross-agglutinating activity of the pure B antibody, 20 ml. volumes of the five sera diluted 1/10 were absorbed for 2 hr. at 37° with

Table 2. *Cross-agglutination reactions of A and B suspensions of* Proteus vulgaris *by anti-B sera*

	A suspensions					B suspensions				
	PR 49	PR 50	PR 51	PR 52	PR 53	PR 49	PR 50	PR 51	PR 52	PR 53
Anti-B serum	Titre									
PR 49	2560	<20	<20	<20	<20	2560	160	640	160	80
PR 50	<20	1280	<20	960	<20	160	640	80	640	<20
PR 51	<20	<20	640	<20	20	320	<20	640	20	<20
PR 52	<20	960	<20	1280	<20	160	480	60	960	40
PR 53	<20	<20	<20	<20	1280	80	20	160	160	2000

homologous A suspension at a final concentration of 10 × 10^9 cells/ml., and after centrifuging the supernatants were tested for residual A and B antibody (Table 4). The absorbed sera show considerably reduced heterologous titres and a diminution in homologous anti-B titre, which is probably due to absorption of homologous A antibody, assuming that the B form carries some A receptor.

Table 3

Antiserum (1/10) absorbed by variant of homologous strain (PR)	Absorbed serum tested against variant	Initial concentration (10^9 cells/ml.) of absorbing suspension of variant A: 160	A: 80	A: 40	A: 20	A: 10	A: 0	B: 160	B: 80	B: 40	B: 20	B: 10	B: 0
49	A	0	0	0	0	tr.	T	T	T	T	T	T	T
49	B	S+	S+	S+	S+	T−	T−	tr.+	S−	S+	S+	T−	T−
50	A	0	0	0	0	0	T	0	S−	tr.+	tr.+	S−	T
50	B	S+	S	S−	S	S+	T−	0	0	0	0	S+	T−
51	A	0	0	0	0	tr.	T−	0	0	0	S	T	T
51	B	T−	T−	T−	T−	T−	T−	0	0	tr.	S	T−	T−
52	A	0	0	0	0	?tr.	T	0	S−	S−	S	S	T
52	B	S+	S+	S+	S+	S+	T	0	0	0	0	0	T
53	A	0	0	0	0	T	T	0	0	tr.	S−	T	T
53	B	T−	T−	T−	T	T	T	0	S−	S−	S	T	T

tr., tr. +, S −, S, S +, T −, T, = increasing degrees of trace, 'standard', and total agglutination; 0 = no agglutination

Table 4

Antiserum to strain (PR)	Titres with A-variant of homologous strain	B-variants of strain (PR) 49	50	51	52	53
49	<20	2560	20	80	80	<20
50	<20	40	240	30	<20	<20
51	<20	160	<20	320	<20	<20
52	<20	nt	nt	nt	40	nt
53	<20	40	<20	120	240	240

nt = no test because of low titre of the antiserum.

The fall in cross-agglutinating titres between B forms after absorption of the sera by A strains which themselves do not show these cross-reactions is, however, more difficult to interpret. The results were confirmed by absorption of these monospecific sera by B variants. Two of these sera only were used, both having a reasonably high homologous titre after absorption. Each was absorbed with falling concentrations of all five B forms, and then tested for homologous B antibody. Only the homologous strain absorbed any antibody.

Stability of variant forms

The strains used for this work were laboratory strains, many of them maintained in stock culture for over ten years, and latterly maintained in the dry state (Stamp, 1947). Nothing is known therefore of the occurrence of the variants described amongst freshly-isolated strains of *P. vulgaris*. B colonies were found exclusively amongst the dry stock cultures. Purified A forms maintained in 0·75 % agar stabs at room temperature remained unchanged in morphology and antigenic constitution. Similar cultures of B forms, however, frequently produced either typical A colonies, or cells with a definite increase in amount of A receptor. This change could also be brought about by frequent subculture either in broth or on MacConkey's plates, the process being slower on solid media. The strain PR 49 in particular often showed as much as 40–50 % reversion from B to A colonies after 2–3 days in agar stabs at room temperature. No quantitative data on this point are available, but these observations suggest that the transition from B to A is more frequent than the reverse. The serological tests of B forms also suggest that the content of non-specific component at the surface may vary, but the velocity and direction of this change is unknown. It is of course possible that the B-antigen complex may arise in type-specific strains masquerading as C variants.

Naming of the variant forms

If the A form of *P. vulgaris* is regarded as modal, and the B and C forms as variants from the mode, the change appears to be mainly A⇄B and A⇄C. There is no evidence of the sequence A→B→C or A→C→B. The variations are reversible; and though the B form has some of the qualities associated with roughness, it is certainly not a completely or an irreversibly rough variant. In these circumstances the least question-begging names for the forms are phases A, B and C. There is a precedent for somatic phases in Leslie & Gardner's (1931) use of four phases to describe stages in the antigenic degradation of *Haemophilus pertussis*; and letters are preferable to numbers, which might suggest a regular sequence of phases, a sequence that was not observed in the A, B and C forms. For convenience, therefore, these forms of *Proteus vulgaris* are provisionally labelled phase A, phase B and phase C.

DISCUSSION

Clearly, *P. vulgaris* can occur in three phases, each characterized by features summarized in Table 5. So far no evidence is forthcoming to suggest that the A and C forms differ antigenically, although it is possible that the latter may sometimes carry B antigen. Their relationship poses a number of interesting questions. Several investigators, studying the swarming of *P. vulgaris* on agar, have described an alternation of bacterial morphology during this process (Klieneberger-Nobel, 1947; Kvittingen, 1949). Short bacilli alternate with long filamentous forms as the culture spreads over the surface of the agar. The C variant may conceivably represent an organism which for some reason has lost the ability to undergo this cyclic change and remains predominantly filamentous.

The change of the modal colony into the B phase is not only related to a change in virulence, but seems also associated with an alteration of antigenic constitution which is clear in general but obscure in detail. The type-specific A phase carries a type-specific antigen. Miles (1951) suggests that this antigen accounts for the toxicity of *P. vulgaris*. It is therefore reasonable to relate the

Table 5. *The characters of the A, B and C phases of* Proteus vulgaris

Phase	Morphology	Mobility	Swarming	Stability in 0·85 % saline after treatment with		
				Nil	HCl	Ethanol
A	Uniform bacillary	+	Step-like	S	S	S
B	Pleomorphic cocco-bacillary chains 'giant'-forms	–	Usually nil	(S)	U	S
C	Uniform filamentous	+	Often in a uniform film	(S)	(S)	(S)

S = stable; (S) = some strains stable, others unstable; U = unstable.

diminished virulence of the B phase to a partial or complete loss of this factor. Whether this is a true loss, with exposure at the cell surface of a new determinant, or a replacement of one by the other, cannot be decided by these investigations. The structure of the antigen appearing in the B phase is uncertain. At first it appeared to be a homogeneous antigen with a diminished group-specificity. The absorption and cross-agglutination tests with B forms suggest more a heterogeneous antigenic surface on these strains. That is, two new antigens are uncovered in the B variant, besides the small amount of A antigen that may still be present. It must be assumed that one of these determinants has so low an antigenic specificity that antibodies to it not only react with similar determinants in a wide range of other strains but also retain some absorptive (or other reactive) affinity for the homologous A antigen and those of other strains. The other confers on the organism a new and narrow specificity. The hypothetical antigenic structures of the two phases may be represented as in Fig. 1. It is possible that the non-specific component reacts with similar components in other bacterial species, since Proteus strains have been described which have receptors in common with Salmonella strains (Maccolini, 1940). Alternatively, this antigenic component may be widespread among certain groups of organisms. Only a small number of variants were examined, and it is quite possible that all the antigenic components of the B variant may be heterogeneous. The small-colony variants described by Felix (1923) may have been B variants. Felix & Rhodes (1931) described them as unstable in saline. The apparent relationship between small-colony variants of an OX 19 strain and a normal OX 2 strain (Weil, 1923) may be explained by the fact that the OX 2 strains used, themselves carried non-specific antigenic receptors. Certainly some OX 2 strains from the National Collection of Type Cultures were markedly unstable in suspension, although I have not tested them for the presence of this receptor. The variant described by Maccolini (1940) produced a small colony, but it was isolated on Wilson & Blair's, not MacConkey's medium, and so may not be comparable. Maccolini states that it was not agglutinated by trypaflavine and was stable in 0·85 and 2·0 % NaCl. This latter test was carried out on living motile suspensions, in which the flagella may have had a stabilizing effect. It was clear, however, that boiled suspensions used for the serology were also fully stable, and in my hands true B variants were rarely entirely stable after boiling.

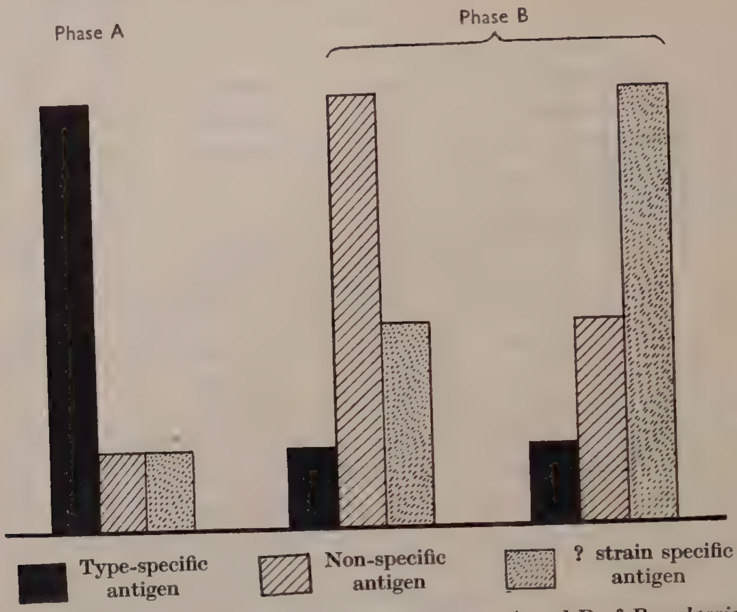

Fig. 1. Diagram of the surface antigens of phases A and B of *P. vulgaris*

The variation A → B shows most of the characters of a partial S → R variation. It was possible, in spite of the fact that all suspensions used were treated with ethanol, that the α-antigen (Stamp & Stone, 1944) may play some part; but all five of the B suspensions failed to agglutinate with an anti-α serum, and it is safe to say that in these cases the phase B antigen complex is not homologous in any of its parts with the α-antigen.

My thanks are due to Dr W. L. M. Perry for the statistical calculations, to Mr F. Welch and Mr Young for the photographs; and Lt.-Col. H. J. Bensted for the anti-α serum.

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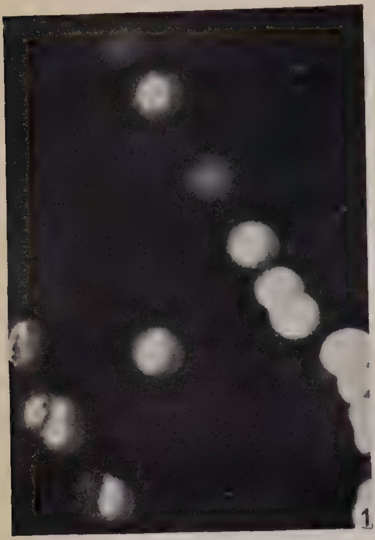

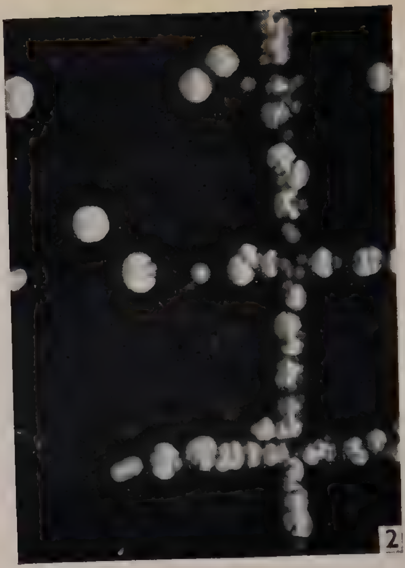

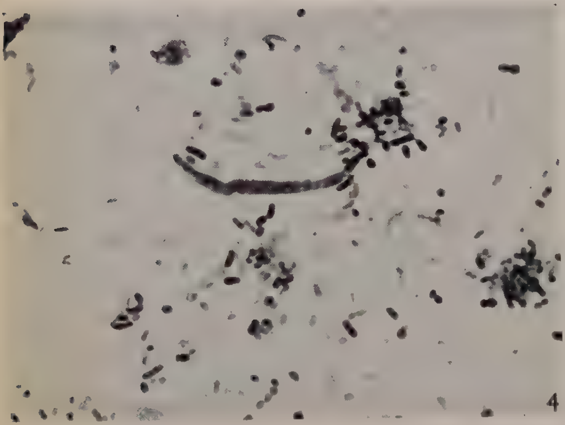

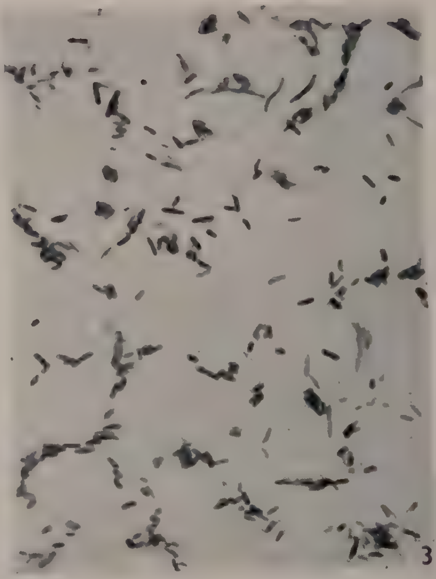

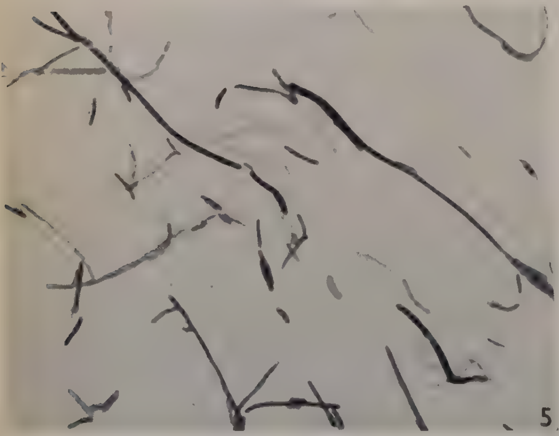

Figs. 1–5

G. BELYAVIN—PHASES OF *PROTEUS VULGARIS*. PLATE 1

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EXPLANATION OF PLATE

Fig. 1 *P. vulgaris*. Twenty-hour-old phase A and C colonies, on MacConkey agar, by oblique transmitted light. (× 4.)

Fig. 2. *P. vulgaris*. Twenty-hour-old phase A and B colonies, on MacConkey agar, by oblique transmitted light. In the confluent growth, colonies in the two phases appear as a mosaic. (× 2.)

Figs. 3–5. *P. vulgaris*. Twenty-hour on MacConkey agar. Methylene blue stain. Phase A phase B and phase C strains respectively. (× 950.)

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Growth and Nisin Production of a Strain of *Streptococcus lactis*

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SUMMARY: Stable mutant colonies were picked from a parent strain of *Streptococcus lactis* which gave improved yields of nisin; further improvement could not be produced by means of colchicine or camphor.

Nisin is stable in the culture fluid at acid pH values, but is inactivated by Seitz filtration or heating at pH 6 to 9. A 'nisinase' was not found.

In the presence of sufficient glucose, growth was increased when the lactic acid formed was neutralized, the concentration of pantothenic acid becoming the limiting factor. Maximum nisin yields were obtained when, during fermentation in the presence of sufficient glucose and pantothenate at 23–30°, the pH was stabilized at *c.* 6, which is the pH optimum for nisin synthesis.

Sodium lactate and nisin are only slightly inhibitory to the organism. Growth comes to an end because of the maximum limiting population density ('M'-concentration) of cells, and is not due to the exhaustion of food or accumulation of toxic products.

Mattick & Hirsch (1944) described an antibiotic substance produced by group N streptococci; clinical applications of this antibiotic were described by Taylor, Hirsch & Mattick (1949) and Hirsch & Mattick (1949). Mattick & Hirsch (1947) described a glucose yeast-extract medium for the production, on a large laboratory scale, of this antibiotic, now called nisin.

The yield of nisin with this culture medium was only *c.* 80 units/ml. (pure nisin contains *c.* 50 units/μg.) and compared unfavourably with that of penicillin or streptomycin. The work now described was designed to increase the yield of nisin and a meat-extract peptone medium replaced the yeast-extract medium.

MATERIALS AND METHODS

Media. For daily subculturing glucose Lemco broth (Mattick & Hirsch, 1947) and for storage bullock's heart medium were used. Evans's peptone (Evans Medical Supplies Ltd., Speke, Liverpool) was used throughout. For growth experiments the following medium was used: peptone 1 %, Lab-lemco 1 %, glucose up to 2·5 %. To the basal medium various additions were made as described below. The vitamin requirements were tested in an acid-hydrolyzed casein medium (Niven, 1944).

The cultures were, unless otherwise stated, incubated at 30° ± 0·1° in conical flasks of 750 ml. capacity and containing 300 ml. of medium inoculated with a 3 % (v/v) inoculum. Values of pH were obtained by means of a glass electrode; optical densities were read in 1 cm. cells with a 'Biochem' absorptiometer (Adam Hilger Ltd., London) using the orange filter. Assays were made by the lag-phase method (Hirsch, 1950).

For work with synthetic resins a 1·5 l. conical flask with an outlet at the

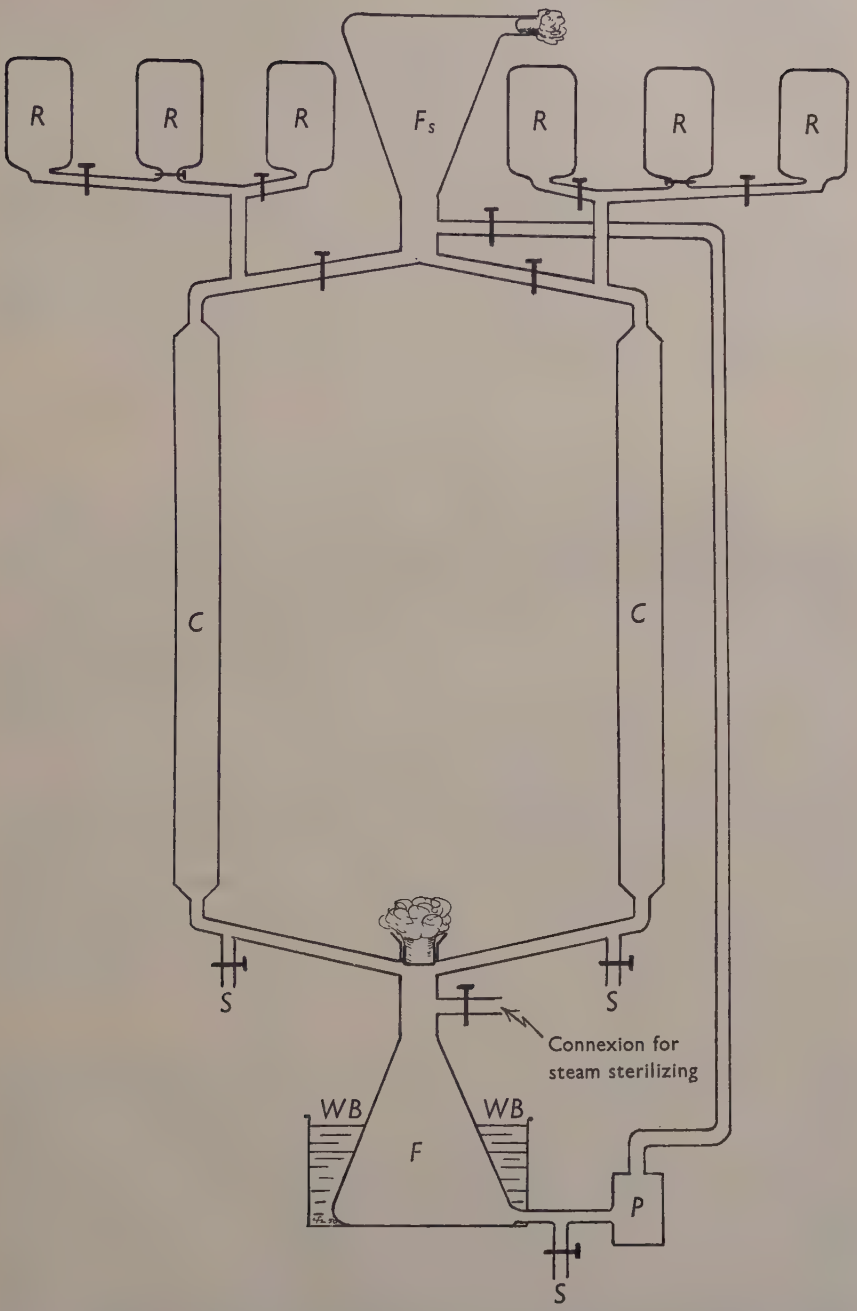

Fig. 1. Apparatus for the removal of nisin and lactic acid by means of synthetic resins from growing cultures of *Strep. lactis* 354/07. F=fermentation vessel; F_s=fermentation vessel storage; P=pump; WB=water-bath; S=sampling points; C=columns for synthetic resins; R=reagents storage for columns.

bottom was used to hold the culture. The culture could be pumped aseptically into a storage vessel and then gravity-fed over columns and returned to the conical flask. The columns could be regenerated or eluted with sterile solvents kept in storage vessels and fed by gravity over the columns (see Fig. 1). *De-acidite E* (Permutit Co. Ltd., London) was washed before use in 50 times its own wet-volume of distilled water, transferred to a column and 1·5 times its volume of 4% (w/v) Na_2CO_3 run over it. It was then washed until the pH of the distilled water was *c.* 10·5. After use the column was regenerated with further additions of 4% Na_2CO_3. *Amberlite I*RC-50 (Analytical Grade, British Drug Houses Ltd., London) was washed with 5 times its own wet-volume of distilled water, columns were made, then treated with two volumes of 0·1 N-NaOH and washed with acetic acid-acetate (0·1 M) buffer at pH 5·0 until no change in the pH of the emerging buffer could be detected.

RESULTS

Properties of the nisin-producing organism

Strain selection. In 1944, strain 354 produced more nisin in milk (which it clotted readily) than in broth; with continued subcultivation in broth this strain lost its ability to clot milk and produced more nisin in broth than in milk. This 'broth trained' strain could not again be made to clot milk or to produce nisin in it. The cultures kept in broth began simultaneously to decline in their ability to produce nisin and the usual concentrations of about 80 units/ml. fell to 20 units/ml. The original milk-cultures were then streaked on Yeastrel glucose agar and single colonies picked. Of twenty-four new strains isolated twenty-one still produced more nisin in milk than in broth, two produced equal amounts in the two media and only one produced more nisin in broth than in milk (354/07). About 2 months after its isolation strain 354/07 was plated and single colonies picked; forty cultures tested were without exception good nisin producers, equalling but not surpassing strain 354/07. This latter strain has now been in use for 6 years without significant change; the work described below was carried out with this strain, which was also used by Berridge (1949).

From the parent strain, different isolates gave different yields of nisin but the high-yielding nisin strain (354/07) selected from it did not. It is not known whether all the strains of 354/07 in use to-day are still homogeneous. Other uncertainties were encountered. One was observed in experiments to find the optimum number of subcultures required to secure maximum nisin production after storage of the producer strain. Usually three to five subcultures were required; sometimes the strains showed a decline after the 8th subculture. At other times one or two subcultures were sufficient and the strain still produced maximal nisin amounts after the 29th subculture.

Attempt to produce variants by treatment with colchicine and camphor. Neither colchicine, camphor or *iso*propylphenylcarbamate induced any changes in growth or nisin production of *Strep. lactis.*

Morphological changes during growth. Polypeptide capsules are known to occur in other species (Bovarnick, 1942). For a while, stained preparations of capsules could be obtained easily with strain 354/07; the strongly Gram-positive cocci were usually enclosed in pairs in a single capsule during the early part of the growth, 'zooglea' becoming more frequent as the culture reached an age of 10–15 hr. Later in this work cultures which showed no capsules were encountered. Examined by phase-contrast microscopy these cultures were observed to multiply; capsules could not be seen, nor could they be stained, but normal amounts of nisin were produced. It may be concluded that the likelihood of nisin being capsular material is small. Some observations of stained preparations (see, for instance, Fig. 2) indicated a change in the capsule (when this was present) corresponding to the appearance of nisin in broth.

Some properties of nisin in culture fluids

The initial pH value of the medium, *c.* pH 7·0, decreased to *c.* 4·6 after 24 hr. growth, the nisin being about equally distributed between the cells and the fluid. Nisin activity was lost during various treatments of the culture fluids. The effect of heating at various pH values is recorded in Table 1, and the further effects of heating and filtration through Seitz-type filter-pads are recorded in Table 2. With heat treatments the results were not clear-cut. Some inactivation may occur at all pH values, but over the range pH 2–4, the rate of liberation of nisin from the cells may equal that of its destruction. There appears to be greater stability at pH 7 than at pH 6. In the culture at the natural final pH, the nisin activity remained unchanged for 10 days at room temperature.

Table 1. *The stability of nisin in culture fluid at various pH values*

(Culture fluid was centrifuged, adjusted to various pH values and boiled for 30 sec.)

pH	Nisin in culture fluid (% of initial value)	Activity lost (%)
2·0	100	0
3·0	78	0
4·0	55	0
4·65*	50	0
5·0	30	0
6·0	*c.* 20	30
7·0	*c.* 20	10 (?)
8·0	17–20	50
9·5	1–10	90

* pH value at end of ordinary fermentation in this medium.

It was of interest to see whether the stability of nisin was also reflected in the absence of a 'nisinase'. A strain of *Bacillus subtilis* used for penicillinase production was strongly inhibited. Species known to be insensitive to nisin, i.e. *Bacterium coli* and *Strep. faecalis*, similarly fail to produce a nisinase. From a sample of dried dung a mould of the genus *Mucor* was isolated which grew on 2 % agar containing 0·5 % crystalline nisin. The activity of the nisin

Table 2. *The inactivation of nisin in cultures subjected to various treatments at different* pH *values*

	Treatments						
		Boiled for				Boiled for 10 sec. then stored 24 hr. at	
pH	Seitz filtration	10 sec.	5 min.	10 min.	20 min.	Room temp.	37°
	Activity lost (%)						
2	0	0	0	0	*c.* 25	—	—
3	27	—	—	0	—	—	—
4	49	—	—	53	—	—	—
5	100	—	—	62	—	—	—
6	100	—	—	*c.* 80	—	*c.* 50	*c.* 85
7	100	—	—	*c.* 80	—	*c.* 85	*c.* 85
8	100	—	—	100	—	*c.* 85–100	*c.* 85–100
9	—	—	—	100	—	*c.* 100	*c.* 100

was diminished by the growth of the mould, but when as little as 0·1 % peptone was incorporated in the medium the mould preferentially used the peptone, the activity of the nisin incorporated into the medium remaining unchanged.

The effect of the composition of the medium and cultural conditions on nisin production

Growth in glucose Lemco peptone broth. Plate-counts, optical density, pH, surface tension, nisin assay and staining reaction were followed continuously in a number of experiments. These observations are recorded in Fig. 2. With increasing numbers of cells there was a corresponding increase in optical density, but the correlation broke down after 8–10 hr. growth; this change appeared to be due not so much to differences between total and viable counts as to the changing morphology of the organism. Considerable metabolic activity preceded the appearance of nisin; the pH fell and the average surface tension decreased from 58 to 54 dynes/cm. In this medium the fermentation was complete in about 8 hr., at which time *c.* 0·7 % glucose out of the initial 1 % had been utilized.

Effect of neutralization of lactic acid formed during fermentation. Rogers & Whittier (1928) and Bernheimer & Pappenheimer (1942) and others have shown that considerably increased growth was obtained when NaOH was periodically added to growing cultures of streptococci to neutralize the acid formed. Similar results were obtained in the present work (see Fig. 3 and 4). Even with the rich glucose peptone Lemco medium, increased amounts of growth (by optical density) were obtained by various additions to the broth, provided that periodic neutralization of acid formed was carried out and that sufficient glucose was present not to become a limiting factor. Liver extract gave the greatest optical density. The nisin production (Fig. 4) was also increased by neutralization, but Ca pantothenate alone was more effective in increasing the yield of nisin than either a mixture of all the known B-vitamins or liver extract.

Microbiological assays on periodically neutralized broth which had initially contained 2·5 % glucose showed that all the pantothenate had been used up.

The effect of buffers. Working with broths at controlled pH (5–6) various buffers were found further to promote the yield of nisin. Citrate and acetate appear to be the most effective single buffers; no specific effect due to acetate only, as was suggested by the work of Guirard, Snell & Williams (1946), was found in this medium. A summary of these findings is given in Table 3. A combination of all the buffers appears to be better than any single buffer. The differences between the average activities of the three groups were found to be significant by the t test ($P = 0{\cdot}01$).

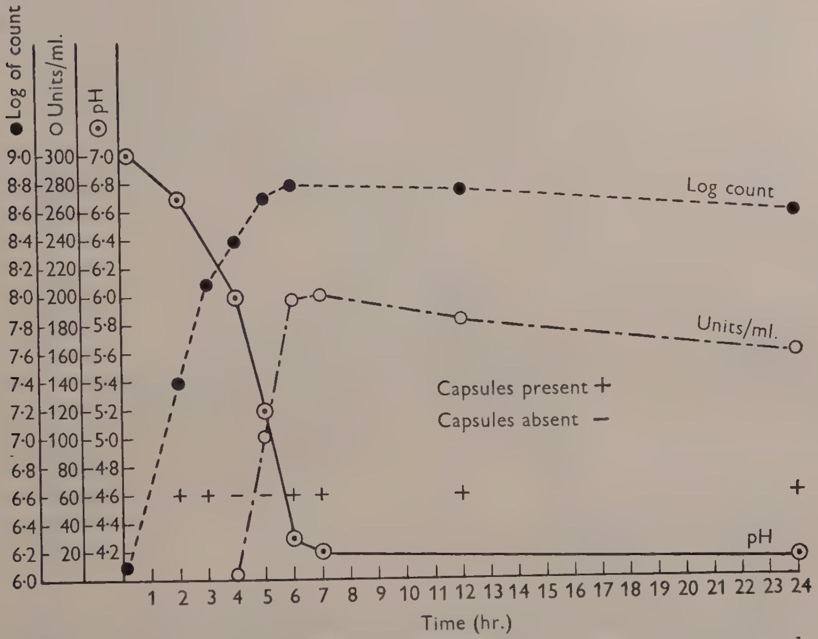

Fig. 2. The growth and nisin production of strain 354/07 in glucose Lemco peptone broth.

It was possible that the increased nisin production was due directly to the accumulation of salts, or that these exercised an indirect effect because of increased osmotic pressure. Experiment showed that increased NaCl or Na_2NO_3 concentrations diminished rather than improved growth and yields of nisin (cf. bottle 4 and 5 with bottle 3, Table 4). It may be that nisin results from a synthesis (see below) and that the three-buffer combination was more effective in maintaining the medium near the optimum pH for nisin production than one or two of the buffers only. Titration curves prepared from media (see Table 3; medium no. 21 and 22 (high activity); 14, 17, 18 and 23 (medium activity); and 15 (low activity)) showed that high buffering power occurred at pH 5·9–6·1 when nisin production also was high. In particular, medium no. 22 showed a marked peak in this pH range.

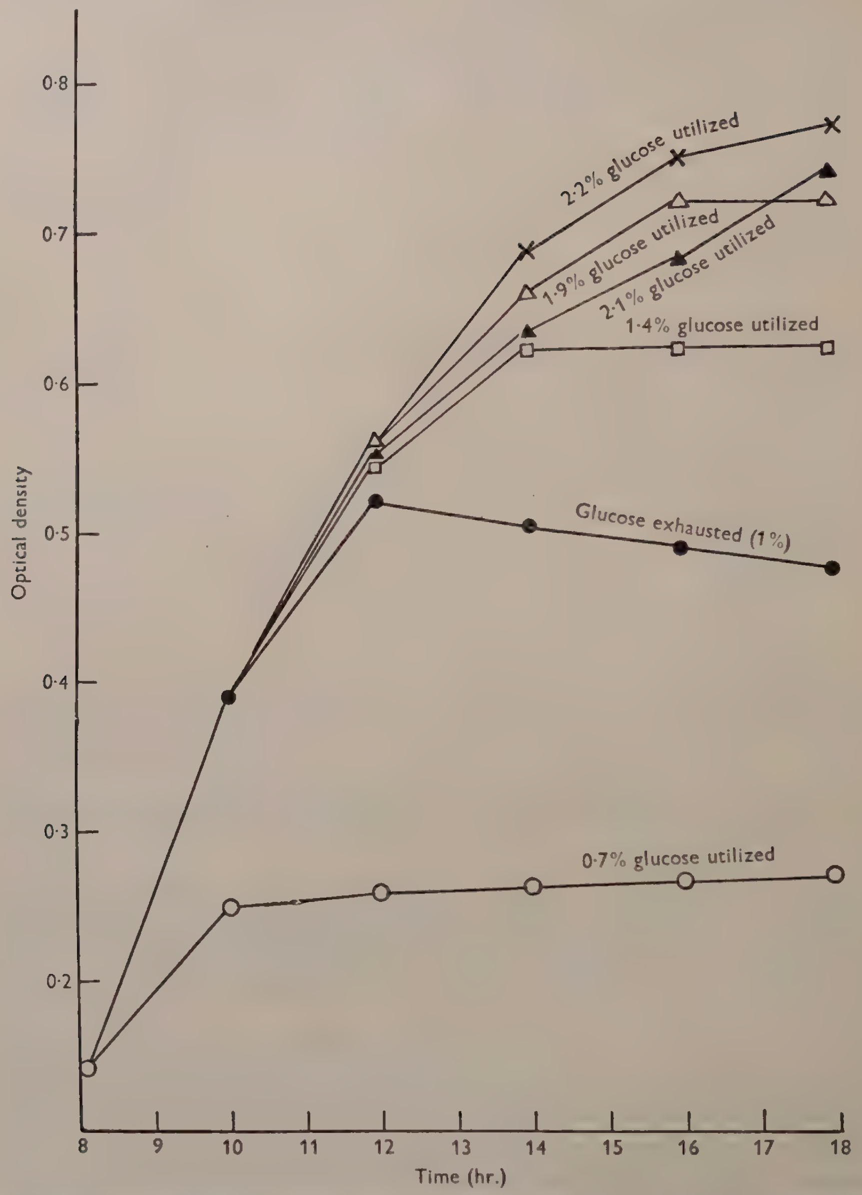

Fig. 3. The effect of certain additions on the growth of *Strep. lactis* as measured by optical density. ○ = medium, normal fermentation; ● = medium, neutralized periodically; □ = glucose initially to 2·5%, neutralized periodically; △ = glucose initially to 2·5% + Ca pantothenate (0·6 μg./ml.), neutralized periodically; ▲ = glucose initially to 2·5% + riboflavin 1 μg./ml., Ca pantothenate 1 μg./ml., nicotinic acid 1 μg./ml., pyridoxin 1 μg./ml., aneurin 0·1 μg./ml., biotin 0·001 μg./ml., xanthine 5 μg./ml., adenine 5 μg./ml., guanine 5 μg./ml., uracil 5 μg./ml., neutralized periodically; × = glucose initially to 2·5% + liver extract, neutralized periodically.

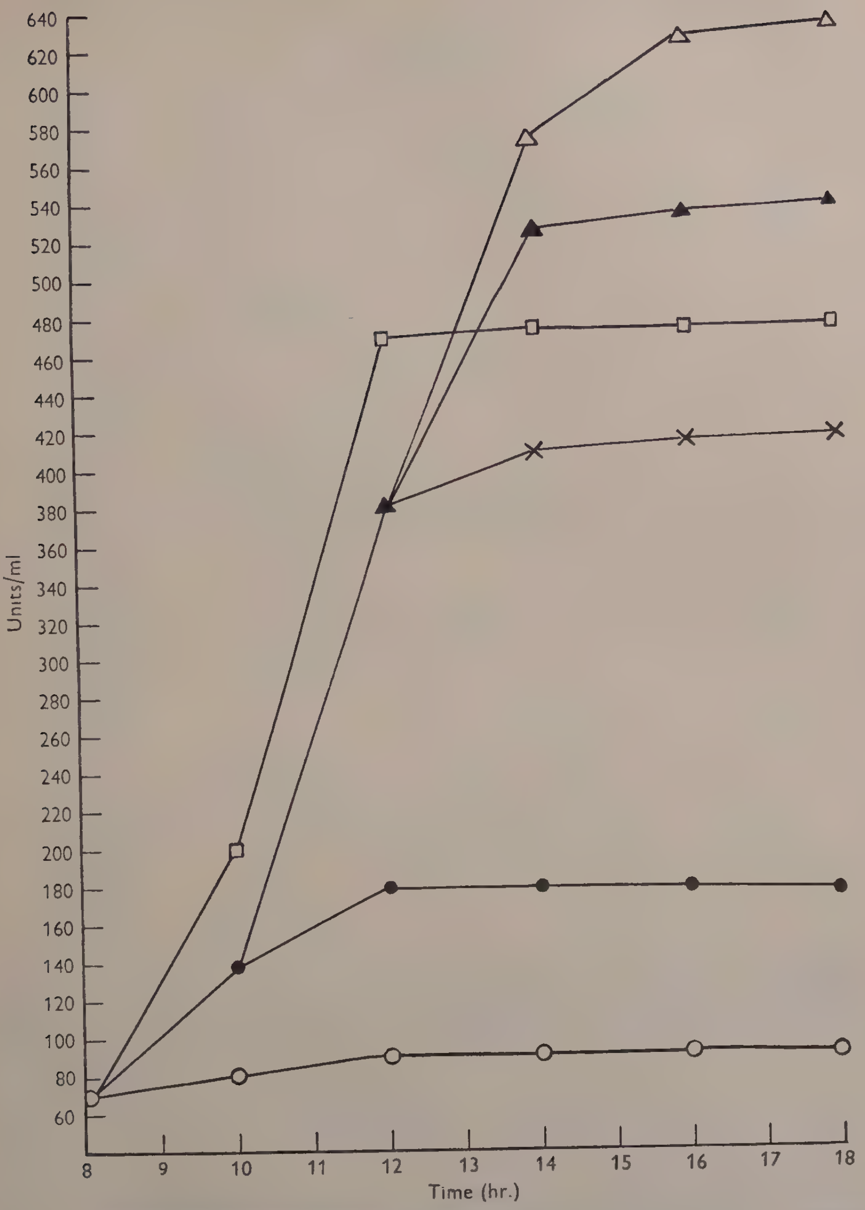

Fig. 4. The effect of certain additions on nisin production by *Strep. lactis*. ○ = medium, normal fermentation; ● = medium, neutralized periodically; □ = glucose initially to 2·5 %, neutralized periodically; △ = glucose initially to 2·5 % + Ca pantothenate (0·6 μg./ml.), neutralized periodically; ▲ = glucose initially to 2·5 % + riboflavin 1 μg./ml., Ca pantothenate 1 μg./ml., nicotinic acid 1 μg./ml., pyridoxin 1 μg./ml., aneurin 0·1 μg./ml., biotin 0·001 μg./ml., xanthine 5 μg./ml., adenine 5 μg./ml., guanine 5 μg./ml., uracil 5 μg./ml., neutralized periodically; × = glucose initially to 2·5 % + liver extract, neutralized periodically.

Table 3. *The effect on nisin formation of the addition of various buffer salts singly and in combinations to media maintained at* pH 5–6 *during growth*

(Ca pantothenate and glucose present in excess.)

Exp. no.	Acetate (%)	Citrate (%)	Phosphate (%)	Yield of nisin (units/ml.)
		Single buffers		
1	0·5	—	—	319
2	1	—	—	401
3	1·5	—	—	664
4	2	—	—	615
5	—	—	0·5	210
6	—	—	1	266
7	—	0·5	—	276
8	—	1	—	527
9	—	1·5	—	771
10	—	2	—	720
			Average yield:	477
		Combinations of two buffers		
11	1	—	0·5	601
12	1	—	1	897
13	1·5	—	0·5	948
14	1·5	—	1	1095
15	1	0·5	—	379
16	1	1	—	955
17	1	1·5	—	1040
18	1·5	1·5	—	1069
19	—	1·5	0·5	815
20	—	1·5	1	783
			Average yield:	858
		Combinations of three buffers		
21	1	1	0·5	1950
22	1·5	1·5	0·5	1963
23	1	1	1	1255
			Average yield:	1723

Table 4 shows that bottle 6 containing only one buffer gave as good a nisin production as bottle 3 containing three buffers, probably because the former was maintained at pH 5·9–6·1 by NaOH additions every ¼ hr. Further, bottle 6 gave much more nisin than bottle 1 (the composition of the medium in the two bottles was identical), bottle 1 was maintained at pH 5–7 by addition of NaOH every 2 hr. The main effect of buffering thus appears to be due to the maintenance of the pH near the optimum. Table 4 also shows that there may be a specific stimulatory effect on nisin production, because more nisin was produced for the same optical density (bottle 8). Table 3 also shows that 2 % of acetate, or citrate, was more than was required for an optimum effect. Similar result was also obtained with 1·5 % phosphate.

The effect of other cultural conditions. The effect of temperature of incubation is shown in Table 5. Maximum growth and nisin production occurred at 25–30°. At 37° growth was more rapid but the final optical density reached was below that at 25–30°. When air was vigorously bubbled into a liquid culture

Table 4. *The effect of salt concentration and regularity of* pH *maintenance during growth on nisin production*

(Strain 354/07 of *Strep. lactis*. Medium adjusted to pH 5–7 except Bottle no. 6 adjusted to 6·0.)

Bottle no.	Composition of medium	NaOH additions each	Final optical density	Yield of nisin (units/ml.)
1	*Basal medium	2 hr.	1·35	615
3	Basal medium + 1·5 % Na citrate + 0·5 % K_2HPO_4	2 hr.	1·25	1660
4	Basal medium + 1·5 % Na citrate + 0·5 % K_2HPO_4 + 1 % NaCl	2 hr.	1·05	1020
5	Basal medium + 1·5 % Na citrate + 0·5 % K_2HPO_4 + 1 % $NaNO_3$	2 hr.	1·15	1070
6	Basal medium	¼ hr.	1·35	1680
8	Basal medium + 1·5 % Na citrate + 0·5 % K_2HPO_4 + 1 % Na_2SO_4	12 hr.	1·15	2130

For effect of salts compare bottle 3, 4 and 5.
For effect of pH compare bottle 1, 3 and 6.
For stimulation of nisin production compare bottles 3 and 8.
* Basal medium was (in %, w/v): Peptone (Evans) 1; Lab-Lemco 1; glucose 2·5; Na acetate 1·5; Ca pantothenate 0·6 μg./ml.

Table 5. *The effect of temperature on growth of, and nisin production by* Strep. lactis, *strain* 354/07

(pH maintained at 5–6 during growth. Ca pantothenate and glucose in excess.)

	Temperature						
	18°	23·6°	24·5°	25·9°	28·5°	30·7°	37°
Time taken to complete fermentation (hr.):	28	25	23	21	20	21	16
At the end of fermentation:							
Optical density:	0·96	1·09	1·10	1·15	1·15	1·12	0·91
Nisin, units/ml.:	369	452	495	542	552	508	386

the growth was delayed and with it the production of nisin, but the end-product appeared normal (cf. Rahn & Richardson, 1942). Nisin production appeared to be normal under strictly anaerobic conditions.

Factors affecting the end of metabolism

After it was found that the pantothenic acid in the medium initially used was exhausted at the end of growth, an excess of Ca pantothenate was always included in the media (0·6 μg./ml. medium). It was possible that the other B vitamins also became exhausted, since even in the rich medium used increased optical densities followed their addition; nisin production was, however, decreased (Fig. 3 and 4). Thus it seems unlikely that nisin production was limited by exhaustion of B vitamins other than pantothenic acid. In agreement with Niven's (1944) findings the results showed that pantothenic acid, riboflavin, nicotinic acid and biotin were essential for growth and that pyridoxin was stimulatory. Growth and nisin production were parallel. It is also possible that a specific nitrogen source becomes exhausted, but this could not be demonstrated. When Lab-Lemco or peptone or both were added to media controlled at pH 6 at the end of the fermentation, neither prolongation of growth nor increase in nisin production resulted.

Three further changes occurred in the medium as the growth proceeded. The surface tension fell from 58 to 54 dynes/cm. This is not of itself enough to stop growth, since according to Ayres, Rupp & Johnson (1923), *Strep. lactis* could grow at a surface tension as low as 40 dynes/cm. Nevertheless, this was one of the unfavourable changes and possibly had an adverse effect.

Table 6. *The effect of sodium lactate on growth of* Strep. lactis, *strain* 354/07

Na lactate added at start		Na lactate added after 6 hr. of growth and cultures then re-incubated for 12 hr.	
Na lactate (%)	Final optical density	Na lactate (%)	Final optical density
0	0·84	—	—
1	0·50	0	0·84
2	0·41	1·25	0·62
3	0·17	2·5	0·53
4	0·09	5	0·30
5	0·04	7·5	0·34
6	0·04	10	0·26

Rogers (1928) was probably the first to describe antibiotic phenomena among the lactic streptococci and concluded that the antibiotic inhibited the organism which produced it. This has to some extent been found in the present work. Using a serial dilution end-point technique about 1000 units/ml. were found to inhibit the producer organism; some 3 years later this experiment was repeated and the self-inhibition titre had risen to about 2000 units/ml. There is little or no effect at 100–200 units/ml., the usual concentration of nisin produced in a culture.

Rogers also investigated the possible limiting effect of lactate (Rogers & Whittier, 1928), but reached no definite conclusion. When lactate was added to growing cultures up to 0·06 M (0·5 %) no differences in the resulting count were detected. As shown earlier in the present work, up to 2·3 % of glucose may be used; this should yield *c.* 4·5 % lactic acid (0·5 M) with this homofermentative strain. Even with this concentration of lactic acid (see Table 6) there was no clear-cut effect. When the lactate was added before growth began, as little as 1 % was sufficient to diminish the amount of growth. At 4–6 % Na lactate growth was virtually stopped, in agreement with the glucose utilization figures above. When lactate was added after the growth had begun, even 10 % of lactate did not completely stop growth, although there was a progressive decrease in the amount of growth with increasing concentrations of lactate, which is therefore much less toxic than might be supposed.

The removal of nisin and lactate from culture fluids

Using the simple apparatus described earlier, growing cultures were pumped over columns of synthetic resin. The use of columns was essential with Deacidite E, which was used for the removal of lactic acid. This resin was found to be unsuitable in a batch process, as apparently acidic substances other than lactic acid were adsorbed and growth was stopped. In a column, however, lactic acid was adsorbed sufficiently specifically or it displaced other components already adsorbed; no interference with growth was observed when a culture-fluid volume of 40 to 3 volumes of resin was used. In a batch process Deacidite E also adsorbed nisin, which could be eluted with weak acids.

A number of synthetic resins was tested for nisin adsorption. Zeocarb 216 (Permutit Co. Ltd., London) adsorbed nisin, the maximum adsorption being obtained when the Zeocarb was used in the H^+ form, but no elution was possible. The Amberlite resins *I*RC–50; *I*RA–400 and *I*R–4B (British Drug Houses Ltd., London) were also tried, of which the first two adsorbed nisin; the resin *I*RC–50 was mainly used. Columns were prepared according to the makers' suggestions for the removal of basic amino-acids. The column was buffered with acetate buffer at pH 4·5–5·0, when about 60–70 % of the nisin was adsorbed, with a culture fluid: resin ratio of 20:1. Nisin was recovered quantitatively by elution with cold 80 % ethanol containing 0·18 % HCl (HCl concentration 0·05 N). Although 100 % recoveries of nisin were obtained, the purity of this resin-adsorbed nisin was disappointing. The activity was only 100,000 units/g. which compared unfavourably with the dried chloroform gel (Mattick & Hirsch, 1947) which had an activity of 500,000–1,000,000 units/ml.

When a growing culture was put over columns of the resin *I*RC–50 further growth was stopped; uninoculated broth treated in the same way no longer supported growth. Therefore this resin was unspecific and essential nutrients as well as nisin were removed from media.

The effect of frothing was then tried. Nitrogen containing 5 % CO_2 was bubbled into a growing culture, the pH of which was maintained by periodically removing the lactic acid over columns of Deacidite E. A 1 in. diameter glass tube, sloping at about 30° was inserted into the top of the conical flask and the rate of bubbling so adjusted that the froth travelled about 5–6 ft. along the glass tube before it was collected. By this means a dry froth having a high nisin activity was obtained. As the fermentation proceeded and the foam produced became less stable, the glass tube was gradually shortened to 3 ft. In the end an average of about 90 % of the nisin was concentrated into about $\frac{1}{10}$th the original volume of culture fluid, the growth in the remaining $\frac{9}{10}$ths of the medium proceeding normally.

The culture fluids thus obtained were free from lactic acid and nisin, but optical densities greater than those obtained by the neutralization technique were not produced. The possibility therefore existed that toxic products other than the above two had also accumulated and were not removed. An experiment to test this point was done. A culture was grown in medium 22 (Table 3) for 4 hr., at which time a perceptible turbidity had appeared. This culture (pH 6·4) was then centrifuged and the cells resuspended in $\frac{1}{10}$th volume of the supernatant clear broth. This concentration corresponded with the highest optical density obtained during fermentations. The concentrated cells were re-incubated for 18 hr. but no further change of optical density resulted; the pH during this time decreased to 4·8. This experiment shows that a maximum limiting population density ('M-concentration', Bail, 1929) is reached with this culture. Provided that there was no exhaustion of the medium and no excess of ionized lactic acid, the M-concentration was the same if (*a*) substances presumably toxic were allowed to accumulate, (*b*) the two known toxic products were removed, (*c*) young cells were concentrated into a small volume of their own unspent medium.

DISCUSSION

Following the observations recorded above a 20–30-fold increase in the yield of nisin has been achieved. In the meat-extract peptone medium used it has been shown that Ca pantothenate becomes exhausted, when the limiting effect of lactic acid is neutralized. It is known that pantothenic acid occurs in co-enzyme A for acetylation and also leads to an increase in pyruvate oxidation (Gunsalus, 1948) and is thus concerned in glycolysis.

There is now little doubt that nisin is a synthetic rather than a breakdown product. Oxford (1945) showed that diplococcin is synthesized by the growing organisms in a medium in which casein hydrolyzed to amino-acids is used. Similar observations were recorded in the course of this work. Low yields of nisin may also be obtained in media containing only amino-acids added individually as such. Moreover, the results in this paper show that the formation of nisin is preceded by considerable metabolic activity and breakdown of glucose. It is possible that the pantothenic acid requirement is connected with this latter process.

It has also been shown that an M-concentration is reached which does not depend on the supply of nutrients or the accumulation of toxic products, a somewhat surprising finding in the case of a vigorous lactic acid-producing organism. This finding rather calls into question the suggestion that the M-concentration is reached because of exhaustion of the oxygen supply (Topley & Wilson, 1946). Rahn & Richardson (1942) found, on the contrary, that vigorous aeration retarded the growth of *Strep. lactis*. It is a reasonable assumption that the underlying principles for the attainment of an M-concentration are the same in *Strep. lactis* (as shown in this work) and in *Shigella* and other organisms (Bail, 1924, 1925).

It appears that no further increases of nisin yields can be expected along the lines of this work, i.e. the production of a maximum number of cells/ml. of culture. That the alternative specific diversion of some enzyme systems into nisin production is possible is shown by the effect of Na_2SO_4 incorporated in the medium. Nisin is known to contain about 5–7 % sulphur (Falconer, 1949).

Little is known about the endo- or exo-cellular nature of nisin. It appears at the moment that an equilibrium between nisin in the cells and nisin in the broth exists, and this depends mainly on pH.

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The Statistical Analysis of Slope-Ratio Assays

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SUMMARY: In the past, the statistical analysis of slope-ratio assays has failed to make specific tests of the validity of assumptions essential to the validity of the conclusions to be drawn, and has instead relied upon a composite test of residual variation. The technique introduced in this paper enables the analysis of variance of assay data to be developed in a manner analogous to that customary for parallel line assays. The paper illustrates the calculations for a 5-point assay, and shows how the analysis of variance, completed with the aid of a set of orthogonal coefficients, gives the required validity tests. Similar analyses for other assay designs, symmetrical and unsymmetrical, are briefly described, and tables of the orthogonal coefficients for 5-, 7-, 9-, and 11-point designs are given. A final section discusses the need for validity tests in biological assays.

In many microbiological assays, the response measured for the test organism shows a linear regression on the dose. The relative potency of two preparations is then estimated by the ratio of the two regression coefficients. The statistical analysis of such a slope-ratio assay was first described in 1945, and improved versions have since been published (Wood, 1946; Finney, 1947). Wood & Finney (1947) discussed the design of slope-ratio assays, and recommended the general adoption of symmetrical $(2k+1)$-point designs: in these, tests are made on 'blanks' (i.e. zero dose) and on k equally spaced doses of the standard and test preparations, using the same replication for all doses. The 5-point design is particularly convenient and useful.

For parallel line assays, in which the response shows a linear regression on the logarithm of dose, tests of the validity of basic assumptions on parallelism and linearity can be made very simply by the use of orthogonal coefficients (Emmens, 1948; Burn, Finney & Goodwin, 1950). The purpose of the present paper is to describe a similar procedure for slope-ratio assays, by means of which the analysis of variance is made much more informative than in the past. In particular, the analysis distinguishes clearly between tests for fundamental and for statistical validity: without these tests the drawing of conclusions from a biological assay is fraught with danger, and without distinction between them the investigator does not know whether an apparently invalid assay needs a modified analysis or is based on unsound premisses.

The 5-point assay

Table 1 records the responses for an assay of riboflavin in a sample of malt (Wood, 1946), expressed in units of 0·05 ml. 0·1 N-NaOH so as to give convenient whole numbers. When the mean responses are plotted against dose (Fig. 1), they are seen to lie close to two straight lines intersecting at zero dose. The statistical analysis will consist in estimating the slopes of the best-fitting lines, and testing their adequacy as a representation of the data.

As Wood showed in an appendix to his paper, the regression coefficients may be obtained by formal estimation of a multiple linear regression equation,

$$Y = a + b_S x_S + b_T x_T.$$

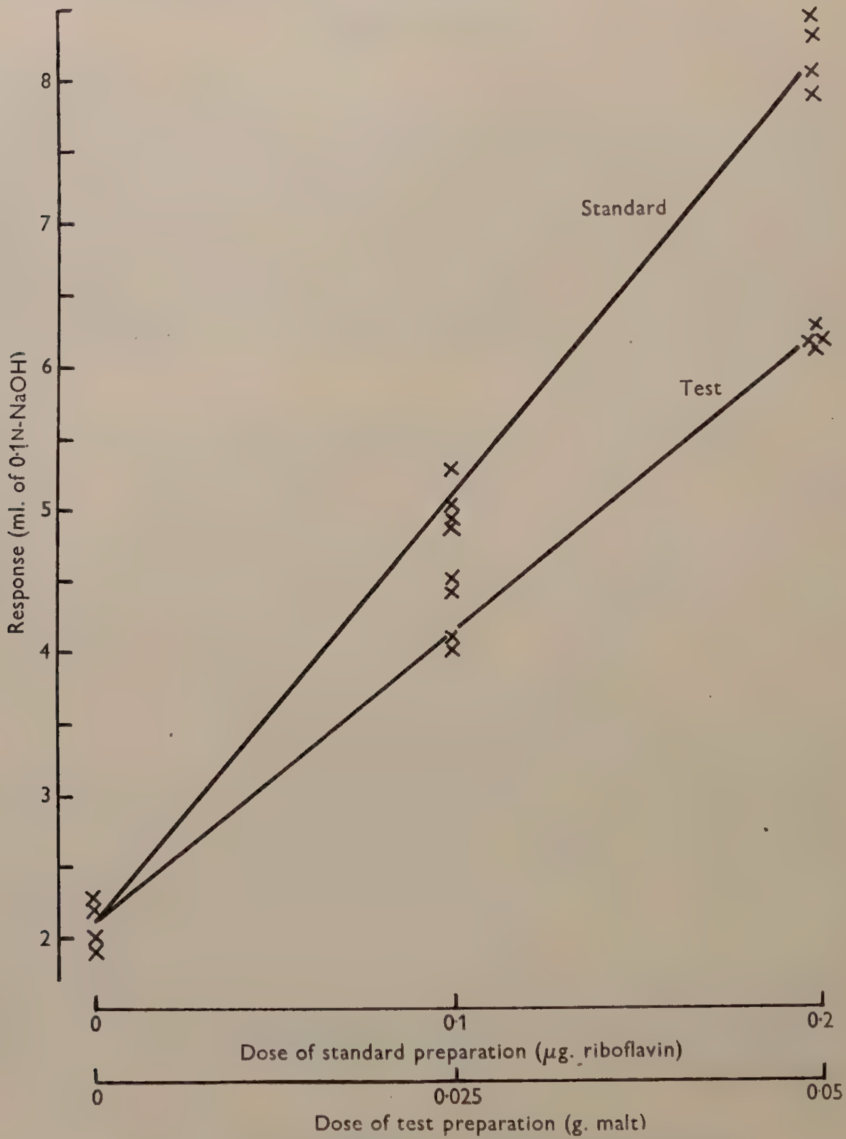

Fig. 1. Response diagram for an assay of riboflavin in malt.

Here x_S represents the dose of the standard preparation, x_T the dose of the test preparation, and Y is the average response to x_S and x_T simultaneously; in ordinary assay practice, the two preparations are always given separately, so that for every response one or other of x_S, x_T is zero. The values of x_S, x_T in the riboflavin assay are shown at the heads of the columns in Table 1. For

Table 1. *Responses in an assay of riboflavin in malt*

One unit of standard preparation = 0·2 μg. riboflavin. One unit of test preparation = 0·05 g. malt. Responses are measured in units of 0·05 ml. 0·1 N-NaOH.

Blanks	Standard		Test	
$x_S=0$	$x_S=\frac{1}{2}$	$x_S=1$	$x_S=0$	$x_s=0$
$x_T=0$	$x_T=0$	$x_T=0$	$x_T=\frac{1}{2}$	$x_T=1$
38	97	167	80	121
45	100	164	88	124
40	105	159	90	122
44	98	156	82	122
167	400	646	340	489

the estimation of the regression coefficients, b_S and b_T (which represent the increase in response per unit increase in dose of the standard and test preparations), sums of squares and products of the deviations of x_S, x_T, y from their respective mean values are needed. Though $S(y-\bar{y})^2$ is not required immediately, it may conveniently be computed at the same time as the others. The calculations of these sums, which are of a type familiar to all who have calculated standard errors and correlation coefficients, proceed as

$$S(x_S-\bar{x}_S)(y-\bar{y})=Sx_Sy-(Sx_S)(Sy)/5n,$$

where $S(\;)$ represents summation over all responses and n is the number of responses measured for each dose (here $n=4$). They may be summarized:

(x_S^2), (x_T^2)	(x_Sx_T)	(x_Sy)	(x_Ty)	(y^2)
5·0	0·0	846·0	659·0	240,198·0
1·8	1·8	612·6	612·6	208,488·2
3·2	−1·8	233·4	46·4	31,709·8

The first two lines correspond to the two terms on the right-hand side of the last equation, the third is their difference. The regression coefficients are the solutions of the equations

$$3\cdot2b_S-1\cdot8b_T=233\cdot4, \quad -1\cdot8b_S+3\cdot2b_T=46\cdot4,$$

and are therefore $\qquad b_S=118\cdot629, \quad b_T=81\cdot229.$

Alternatively, b_S and b_T can be calculated directly from the five dose totals at the foot of Table 1: if the dose totals be denoted by C (for the blanks), S_1, S_2, T_1, T_2, then

$$b_S=\frac{2}{35n}(-15C+S_1+17S_2-6T_1+3T_2),$$

and

$$b_T=\frac{2}{35n}(-15C-6S_1+3S_2+T_1+17T_2),$$

which give the same numerical values as before. The advantage in this form of calculation is slight, however, because $S(x_S-\bar{x}_S)(y-\bar{y})$ and $S(x_T-\bar{x}_T)(y-\bar{y})$ are required in the construction of the analysis of variance.

The analysis of variance (Table 2) must next be completed. The total sum of squares of deviations for the responses is easily divided into components between doses (4 degrees of freedom) and within doses ($\overline{n-1}$ degrees of freedom from each dose); the sum of squares between doses is calculated from the five dose totals, in the usual manner. The quantity

$$b_S S(x_S-\bar{x}_S)(y-\bar{y})+b_T S(x_T-\bar{x}_T)(y-\bar{y})=118{\cdot}629\times 233{\cdot}4+81{\cdot}229\times 46{\cdot}4$$
$$=31{,}456{\cdot}9$$

is the part of the sum of squares between doses accounted for by the regression. The remainder may be split into two components, with 1 degree of freedom each, the first dependent upon the deviation of the mean response for the blanks from regressions fitted to the non-zero doses and the second dependent upon the deviation of the point of intersection of regressions fitted separately to the two preparations from the zero level of dose. The first, to be described as *Blanks*, is calculated as $L_C^2/14n$, where

Table 2. *Analysis of variance for the riboflavin assay*

Adjustment for mean 208,488·2

Nature of variation	D.F.	Sum of squares	Mean square
Regression	2	31,456·9	—
Blanks	1	2·2	2·2
Intersection	1	34·2	34·2
Between doses	4	31,493·3	—
Error	15	216·5	14·43
Total	19	31,709·8	—

$$L_C=2C-2S_1+S_2-2T_1+T_2,$$

provides a test of statistical validity, in particular of whether the assumption of linear regression down to $x_S=x_T=0$ is contradicted by the data. The second, to be described as *Intersection*, is calculated as $L_C'^2/10n$, where

$$L_C'=2S_1-S_2-2T_1+T_2,$$

provides a test of fundamental validity of the assay, for unless regressions fitted separately to the standard and test data intersect at $x_S=x_T=0$ (except for deviations attributable to random variation) the whole basis of the assay is faulty (cf. Jerne & Wood, 1949). For the data of Table 1,

$$L_C=-11, \text{ with divisor } 56,$$

and

$$L_C'=-37, \text{ with divisor } 40.$$

The three components, regression, blanks, and intersection, add exactly to the sum of squares between doses.

In this instance, neither the blanks nor the intersection component is significantly greater than the error mean square, and estimation of the relative potency may proceed. One unit of the test preparation is estimated to contain $R=b_T/b_S=0{\cdot}685$ units of the standard. Fiducial limits may be found by formulae described elsewhere (Wood, 1946; Finney, 1947), and are 0·646, 0·724 for the 95% probability level. Reference to the definition of units at the head of Table 1 then shows that 1 g. malt is estimated to contain 2·74 μg. riboflavin, with 95% fiducial limits at 2·58 and 2·90 μg.

Wood (1946) proposed to use the deviations of the quantities

$$L_S = C - 2S_1 + S_2 \quad \text{and} \quad L_T = C - 2T_1 + T_2$$

from zero as tests of validity, these representing tests of the linearity of regressions for each preparation separately, using the blanks in turn with each. The method proposed here, though based upon the general procedure described below, is equivalent to testing $(L_S + L_T)$ and $(L_S - L_T)$. The advantages for the present proposal are that it separates the tests into one of statistical and one of fundamental validity, that it fits into the structure of the analysis of variance, and that it generalizes to the $(2k+1)$-point design. In practice, the decisions taken in respect of any particular set of data will usually be the same if based on Wood's analysis instead of on that advocated here.

Other assay designs

The scheme for the analysis of the sum of squares between doses which has been illustrated for the symmetrical 5-point assay may be extended to other designs. For the 9-point design, for example, having n tests at four doses of each preparation as well as for the blanks, the sum of squares for blanks is $L_C^2/28n$, where

$$L_C = 4C - 2(S_1 + T_1) - (S_2 + T_2) + (S_4 + T_4),$$

and that for intersection is $L_C'^2/12n$, where

$$L_C' = 2(S_1 - T_1) + (S_2 - T_2) - (S_4 - T_4).$$

These, together with the sum of squares for regression found in the usual manner, account for 4 of the 8 degrees of freedom between doses. The remainder can be partitioned into quadratic and cubic components for each preparation separately, using contrasts

$$S_1 - S_2 - S_3 + S_4 \quad \text{and} \quad T_1 - T_2 - T_3 + T_4$$

with divisor $4n$, and

$$-S_1 + 3S_2 - 3S_3 + S_4 \quad \text{and} \quad -T_1 + 3T_2 - 3T_3 + T_4$$

with divisor $20n$. The coefficients for these are the same as are used for quadratic and cubic terms in the more familiar parallel line assays, except that the two preparations are examined separately. For most purposes, a composite test of the residual curvature components comprised in these 4 degrees of freedom would suffice, but the full analysis may sometimes assist the understanding of a deviation from linearity.

The general formulae for a symmetrical $(2k+1)$-point assay, with n responses measured for each dose, have been developed and will be described elsewhere. For $k=1$, the calculations are trivial, as the regression coefficients are found from the differences between the responses to the preparations and that for the blanks; Wood & Finney (1947) have advised against the use of this design because of its complete lack of validity tests. Tables 3–6 assist the calculations for designs with $k = 2$, 3, 4, or 5, and their use will be understood by comparing them with the instructions already given for the 5-point and 9-point designs. The regression coefficients are to be found exactly as described for the riboflavin assay (first method), by forming sums of squares and products and solving two linear equations. The expression

$$b_S S(x_S-\bar{x}_S)(y-\bar{y})+b_T S(x_T-\bar{x}_T)(y-\bar{y})$$

always gives the sum of squares (2 degrees of freedom) for the regression. From the dose totals are calculated the blanks and intersection components as well as components for quadratic, cubic, and quartic curvature (where these can be formed). Provided that the assay is not demonstrated to be invalid, formulae below the tables may be used to assess the precision of $R\ (=b_T/b_S)$, the estimate of relative potency. The first two equations give the variances of b_S and b_T and their covariance, and the third gives the variance of R. In these formulae, s^2 is the error mean square in the analysis of variance and b_S, b_T are supposed to relate to scales of measurement for doses that make the highest dose of each preparation equal to 1 unit. The formula for $V(R)$ is applicable only if $b_S^2/V(b_S)$ is large (say greater than 100); if this condition is not satisfied, fiducial limits to R must be found by formula (32) given by Finney (1947).

Table 3. *Orthogonal coefficients for a 5-point slope-ratio assay*

	Blanks	Standard		Test		
Component	C	S_1	S_2	T_1	T_2	Divisor
L_C	2	−2	1	−2	1	$14n$
L'_C	0	2	−1	−2	1	$10n$

$$V(b_S)=V(b_T)=\frac{64s^2}{35n};\ \mathrm{Cov}\,\{b_S,\,b_T\}=\frac{36s^2}{35n};\ V(R)=\frac{8s^2(8-9R+8R^2)}{35nb_S^2}.$$

Table 4. *Orthogonal coefficients for a 7-point slope-ratio assay*

	Blanks	Standard			Test			
Component	C	S_1	S_2	S_3	T_1	T_2	T_3	Divisor
L_C	6	−4	−1	2	−4	−1	2	$78n$
L'_C	0	4	1	−2	−4	−1	2	$42n$
Quadratic (S)	0	1	−2	1	0	0	0	$6n$
Quadratic (T)	0	0	0	0	1	−2	1	$6n$

$$V(b_S)=V(b_T)=\frac{279s^2}{182n};\ \mathrm{Cov}\,\{b_S,\,b_T\}=\frac{81s^2}{91n};\ V(R)=\frac{9s^2(31-36R+31R^2)}{182nb_S^2}.$$

The same principle may be applied to the analysis of unsymmetrical assays, such as that first used as an illustration of the slope-ratio procedure (Finney, 1945, 1947). The square for blanks is then most easily found by introducing an auxiliary variate x_O which takes the value 1 for all blank responses, O for all others; x_O is used formally as though it were a measure of dose for a third 'preparation'. The difference between the sum of squares with 3 degrees of freedom for a multiple regression on x_O, x_S, x_T, and that with 2 degrees of freedom for the regression on x_S, x_T is the square for blanks. A sum of squares with 4 degrees of freedom may be formed by finding the sum of squares (2 degrees of freedom) between blanks, the total of all responses to the standard preparation, and the total of all responses to the test preparation, and adding to it squares for two linear regressions calculated from each preparation independently, ignoring the blanks; the difference between this and the sum of squares with 3 degrees of freedom for regression on x_O, x_S, x_T is the square for intersection. The equivalence of this apparently arbitrary procedure to that used earlier may be demonstrated by elementary (though laborious) algebra.

Table 5. *Orthogonal coefficients for a 9-point slope-ratio assay*

	Blanks	Standard				Test				
Component	C	S_1	S_2	S_3	S_4	T_1	T_2	T_3	T_4	Divisor
L_C	4	−2	−1	0	1	−2	−1	0	1	$28n$
L'_C	0	2	1	0	−1	−2	−1	0	1	$12n$
Quadratic (S)	0	1	−1	−1	1	0	0	0	0	$4n$
Quadratic (T)	0	0	0	0	0	1	−1	−1	1	$4n$
Cubic (S)	0	−1	3	−3	1	0	0	0	0	$20n$
Cubic (T)	0	0	0	0	0	−1	3	−3	1	$20n$

$$V(b_S)=V(b_T)=\frac{136s^2}{105n};\ \mathrm{Cov}\,\{b_S,\,b_T\}=\frac{16s^2}{21n};\ V(R)=\frac{8s^2(17-20R+17R^2)}{105nb_S^2}.$$

Table 6. *Orthogonal coefficients for an 11-point slope-ratio assay*

	Blanks	Standard					Test					
Component	C	S_1	S_2	S_3	S_4	S_5	T_1	T_2	T_3	T_4	T_5	Divisor
L_C	20	−8	−5	−2	1	4	−8	−5	−2	1	4	$620n$
L'_C	0	8	5	2	−1	−4	−8	−5	−2	1	4	$220n$
Quadratic (S)	0	2	−1	−2	−1	2	0	0	0	0	0	$14n$
Quadratic (T)	0	0	0	0	0	0	2	−1	−2	−1	2	$14n$
Cubic (S)	0	−1	2	0	−2	1	0	0	0	0	0	$10n$
Cubic (T)	0	0	0	0	0	0	−1	2	0	−2	1	$10n$
Quartic (S)	0	1	−4	6	−4	1	0	0	0	0	0	$70n$
Quartic (T)	0	0	0	0	0	0	1	−4	6	−4	1	$70n$

$$V(b_S)=V(b_T)=\frac{380s^2}{341n};\ \mathrm{Cov}\,\{b_S,\,b_T\}=\frac{225s^2}{341n};\ V(R)=\frac{10s^2(38-45R+38R^2)}{341nb}.$$

Validity tests

The reader may wonder what is the use of this apparently complicated analysis, and may inquire whether it is really necessary. Certainly it adds nothing to the precision of the estimate of potency to be quoted as the conclusion from an assay. Any conclusion, however, rests upon certain assumptions about the nature of the preparations assayed and the character of the dose-response relationship (Jerne & Wood, 1949). If these assumptions are untrue, the assay may be fundamentally invalid, or its statistical analysis may need radical alteration before any conclusion can legitimately be drawn. The experienced statistician may often be able to dispense with explicit tests of the validity of these assumptions, because he can see by inspection of the data that no significant evidence of invalidity is present; nevertheless, he must have at his command methods of testing for use in critical cases where he cannot trust his judgement. The user of assay techniques whose statistical experience is limited needs to be more cautious about the acceptance of basic assumptions without explicit and objective tests; for him especially, validity tests such as have been described may be important and frequently used. Indeed, validity tests should be regarded as an essential feature of biological assays, never to be neglected merely because they are laborious; the only excuse that can rightly be made for omission of explicit tests is that the results of these could be foretold with certainty, without performing the arithmetical operations. Though the statistical analysis of slope-ratio assays is more laborious than that of parallel line assays, adoption of a symmetrical design eliminates much of the labour and is theoretically desirable; moreover, the labour lies chiefly in the calculation of the regression coefficients, and, for the more important symmetrical designs, as shown above, the validity tests can be based on standard sets of orthogonal coefficients just as easily as for parallel line assays.

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Significance of Calcium in the Fruiting of *Chaetomium* Species, Particularly *Chaetomium globosum*

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SUMMARY: Calcium is required by *Chaetomium globosum* for the normal formation of perithecia, but is not essential for vegetative growth. Calcium is at least partly replaceable by strontium or barium. Most of the other species of *Chaetomium* tested responded to calcium, either in fruiting or growth, or both.

Suitable doses of calcium reproduce in a large measure the previously observed effect of jute extract on the fruiting of *Chaetomium* species. The presence of calcium in jute extract was established.

It has been observed previously that growth and perithecial formation of *Chaetomium globosum* are markedly stimulated by a hot-water extract of jute fibre (Buston & Basu, 1948), and that the ash of this extract also has a somewhat similar effect on this and other species of *Chaetomium* (Basu & Bose, 1950). The specific nature of the factors responsible was not established. Further investigation, embodied in the present paper, shows that calcium, which is present in jute extract, stimulates the fruiting of *C. globosum*. Light has also been thrown on the effect of some other trace metals on this fungus, and of calcium on the growth and fruiting of a few other *Chaetomium* species.

MATERIALS AND METHODS

Three strains of *C. globosum* Kunze, all isolated in this laboratory, were used in these studies; no. 79, the strain used in our previous investigations, and no. 79·2, were isolations from perithecial growths on paper, and no. 79·1 was from a plating of fresh jute fabric. Stock cultures of these were preserved on malt agar slants in the refrigerator.

The medium used was Czapek's salt solution (with K_2HPO_4 and without Fe) and 0·2 % glucose (w/v); pH was 7·4. Ten ml. quantities were distributed into 125 ml. American made (see below, p. 236) Pyrex conical flasks (cleaned before each test with hot chromic-sulphuric acid) and sterilized at 10 lb. for 10 min. Each flask was inoculated with a loopful of spore suspension prepared by crushing a few perithecia in a drop of sterile distilled water. Incubation was at 27° for 20 days.

Structures representing the first beginning of perithecia formation and only just visible as small specks on the mycelium were recorded as immature perithecia. The appearance of ascospores was detected by microscopical examination of crushed perithecia. Counting of perithecia was often difficult and probably not very accurate when the number was very large or when they tended to adhere in thick clusters. Size of perithecia was computed by eye; perithecia reported as 'large' had the dimensions demanded by the species description;

however, smaller but mature perithecia were always indistinguishable from these except in size.

Usually duplicate flasks were used and both results are reported; lack of resources did not always permit use of flasks in pairs, but the positive results reported have been checked by repeated experiments. Though replications did not always yield the same figures and sometimes showed considerable divergences, results have not been considered valid unless the same relative values were obtained.

RESULTS

Strain 79 formed perithecia even when the initial glucose concentration was 0·01 % (w/v), although their number was limited by the small amount of growth; at 0·1 % (w/v) glucose a few small but mature perithecia were formed, but at 0·2 % (w/v) glucose only a number of immature perithecia, seldom forming spores in any numbers although often possessing long and characteristically curved terminal hairs, were to be seen.

Effect of calcium

The effect of Ca (as $CaCl_2$) over a wide range of concentrations is shown in Table 1. At the two highest concentrations there was some turbidity in the medium when first prepared due to the precipitation of Ca salts, but this soon disappeared in the course of growth. With each strain the best all-round effect on fruiting was obtained at 10 p.p.m. Ca. The effect disappeared at 0·1 p.p.m. The inhibitory action of comparatively high Ca concentrations was manifested, apart from the results recorded in Table 1, in the incomplete growth of strain no. 79 at 1000 p.p.m. and in the texture of the mycelia of strains nos. 79·1 and 79·2 which formed small detached colonies instead of the normal mat. It was also noticed that as the Ca concentration reached 10 p.p.m. perithecia tended to adhere in clusters, their size was more uniform, and there were few immature perithecia left. The proportion of immature perithecia increased particularly with the lowering of Ca concentration. Freedom of perithecial formation was noticeably higher in strain no. 79·1 than in the other two strains. Strain no. 79 displayed the sharpest responses to Ca so that this strain was used in more experiments than the others.

The optimum Ca requirement was more accurately determined within a narrower range of concentrations and was found to be near 10 p.p.m. for all strains. The results obtained with strain no. 79 are given in Table 2. It may be noted that perithecial number and size of strain no. 79 at 10 p.p.m. Ca level are different in Tables 1 and 2. The same tendency, namely that of an increase in number at the expense of size, or vice versa, was observed, within limits, in several other instances.

Other trace metals

The presence of Ca in jute extract was established by a microchemical test using a saturated solution of picrolonic acid which gave rectangular crystals characteristic of Ca. A spectrographic analysis of the ash of jute extract was kindly made for us by Prof. P. B. Sarkar of the Calcutta University and, besides

Table 1. *Effects of different concentrations of calcium on three strains of* Chaetomium globosum

Strain no.	Ca (p.p.m.)	Day of			Mature perithecia after 20 days	
		First visible growth	Appearance of immature perithecia	Appearance of ascospores	No.	Size
79	0	2, 2	9, 10	>20, >20	0, 0	—
	0·1	2, 2	9, 10	>20, >20	0, 0	—
	1	2, 2	7, 10	11, 12	120, 120	Small, medium
	10	2, 2	6, 10	10, 12	200, 200	Large
	100	3, 4	10, 11	12, 13	200, 200	Small, medium
	1000	9, 10	>20, >20	—	0, 0	—
79·1	0	2, 2	8, 10	17, 17	80, 55	Small, medium
	0·1	2	8	17	85	Small, medium
	1	2, 2	6, 6	11, 12	200, 180	Small, medium
	10	2, 2	5, 6	7, 8	300, 250	Medium, large
	100	3, 3	7, 8	10, 10	250, 225	Small, medium, large
	1000	5, 7	15, 17	17, 20	80, 3	Medium
79·2	0	2, 2	>20, >20	—	0, 0	—
	0·1	2, 2	10, 16	13, >20	10, 0	—
	1	2, 2	10, 12	12, 13	120, 100	Medium
	10	2, 2	6, 6	9, 10	180, 100	Medium, large
	100	3, 3	9, 11	12, 13	70, 50	Small, medium, large
	1000	5, 6	17, 20	19, >20	22, 0	Medium

Table 2. *Optimum calcium concentration for* Chaetomium globosum *strain* 79

Ca (p.p.m.)	Day of appearance of		Mature perithecia after 20 days	
	Immature perithecia	Ascospores	No.	Size
0	7	>20	0	—
1	5	8	90	Small
5	5	7	150	Medium
6·7	5	7	300	Medium
10	5	7	330	Medium
20	6	7	160	Medium
100	10	12	120	Small

Table 3. *Comparative effects of some other metals on* Chaetomium globosum *strain* 79

Metal	Concn. (p.p.m.)	Day of appearance of		Mature perithecia after 20 days	
		Immature perithecia	Ascospores	No.	Size
Ba	10	5, 6	8, 9	110, 90	Medium, large
	1	6	9	60	Small, medium
Sr	10	6, 6	8, 9	250, 170	Medium, large
	1	6	9	40	Small
Mn	10	6, 6	10, 10	30, 20	Minute
	1	6	10, 11	30	Small
Ca	10	5, 5	8, 9	300, 200	Medium, large
	1	5	9	120	Small, medium

confirming the presence of Ca, it indicated the occurrence of Mg, Fe, Co, Sn, Ni, Mn, Pb, Si and P in the ash. Since Mg and P were present in the basal medium, only the other elements (with the exception of Si) were tested, mostly at 10 and 1 p.p.m. levels, with strain no. 79. No positive effect was noticed except a small one with Mn, already mentioned (Basu & Bose, 1950). Co and Ni had inhibitory action on vegetative growth. Mg in the medium could not be replaced by Ca; without Mg, growth was small and there was no fruiting even in the presence of Ca which only slightly increased growth. Ca was found to be replaceable, at least partly, by Ba and Sr. These elements and Mn are compared with Ca in Table 3. The salts used were $BaCl_2$, $Sr(NO_3)_2$ and $MnSO_4.4H_2O$, recrystallized before use. In other experiments Ba and Sr at high concentrations exhibited the same inhibitory effect as observed with Ca. The perithecial number given by Ba was never as high as that obtained at optimum Ca concentration. Ba and Sr had about the same optimum concentration as Ca, but Mn showed the best effect at *c.* 1 p.p.m. Even at this concentration the perithecial number and size were never comparable with those in presence of Ca, although the small perithecia given became mature and developed spores. When Ca was combined with one, two or three of the other metals at optimum concentrations no superiority over Ca alone was observed.

Table 4. *Effects of jute extract on the three strains, and comparative effects of jute extract, its ash, and calcium on strain* 79 *of* Chaetomium globosum

		Day of			Mature perithecia after 20 days	
Strain no.	Supplement	First visible growth	Appearance of immature perithecia	Appearance of ascospores	No.	Size
79	Ca (10 p.p.m.)	2, 2	6, 6	8, 11	300, 250	Medium, large
	Jute extract ash*	2, 2	6, 6	9, 11	300, 250	Medium, large
	Jute extract*	1, 1	5, 5	7, 9	500, 400	Medium
	Jute extract* + Ca (10 p.p.m.)	1, 1	5, 5	7, 9	500, 300	Medium, large
79·1	Jute extract*	1, 1	4, 5	7, 8	300, 300	Medium, large
79·2	Jute extract*	1, 1	4, 5	7, 9	300, 300	Medium, large

* Concentration of jute extract was equivalent to 3·5 g. jute fibre/100 ml. of final medium.

Comparison with jute extract

Combinations of Ca with biotin, aneurin, riboflavin, pyridoxin, nicotinic acid or pantothenic acid (all present in jute extract; Buston & Basu, 1948), at 1, 0·1, 0·01 μg. of each vitamin per flask also did not yield better results. The effect of Ca on fruiting, though somewhat different from that of jute extract, was nearly the same as the effect of the ash of this extract at the concentration tried (Table 4). Stimulation of germination and growth by jute extract was evident in all three strains, and jute extract also gave an appreciably higher number of perithecia than Ca in strains nos. 79 and 79·2. However, in the case of strain no. 79 the perithecial size generally obtained with jute extract did not appear to be quite up to normal size. This was confirmed by measurement of a large number of perithecia from several different sets of experiments. Perithecia obtained in presence of Ca usually exceeded 200 μ. in length, whereas those given by jute extract rarely did so. This is another example of the balance between perithecial number and size as observed above. Such differences, if any, were much less pronounced in strains 79·1 and 79·2.

Comparison of growth and fruiting effects

Ca stimulated fruiting in two strains even when added to stale medium long after vegetative growth had ceased and autolysis had set in (Table 5). The absence of response by strain no. 79·2 may be connected with the absence of immature perithecia before the addition of Ca (Table 1). The number of perithecia produced by strain no. 79 was smaller than when Ca was added before growth, probably due to the presence of products of staling and autolysis. However these results indicated that fruiting could be stimulated independently of vegetative growth. The same conclusion is suggested also by the effect of jute extract.

Table 5. *Effects on the fruiting of the three strains of* Chaetomium globosum *of supplements added after completion of growth*

Ca added at 10 p.p.m. where used.

Strain no.	Mature perithecia after 20 days		Supplement added after 20 days	Mature perithecia after 40 days	
	No.	Size		No.	Size
79	0, 0	—	Ca	120, 60	Medium
79	4	Small	Jute extract*	120	Medium
79·1	80, 55	Small to medium	Ca	300, 300	Medium
79·2	0, 0	—	Ca	0, 0	—

* Concentration of jute extract as in Table 4.

The effects of jute extract and Ca on vegetative growth were compared with growth on the basal medium by progressive measurement of mycelium weights till growth had reached the maximum. Fig. 1 shows that Ca had no significant effect on growth although jute extract increased both rate of growth and the maximum mycelium weight reached. Since growth on jute extract alone in distilled water was negligible (although some mature perithecia were formed), the increased mycelium weight given by jute extract + basal medium cannot be explained by the presence of additional carbon sources in the extract. That Ca did not have any perceptible influence on germination, rate of growth or cell synthesis efficiency was confirmed in another experiment in which both increase in mycelium weight and disappearance of glucose in the medium was progressively followed with and without 10 p.p.m. Ca in the medium; in this experiment the pH reached 9·5 shortly after growth started in both media and remained more or less stationary there.

Effect of calcium on other species

The effect of Ca at 10 p.p.m. concentration was tried on several other species of *Chaetomium*. The results are given in Table 6, where instances of stimulation of germination, of ultimate growth and of fruiting are seen. *C. microcephalum* Ames (no. 113) and *C. pachypodioides* Ames (no. 114), which were also tested, did not germinate even in the presence of Ca. It should be noted that only those species in which fruiting is stimulated by Ca had previously shown a similar response to jute extract or its ash (Basu & Bose, 1950).

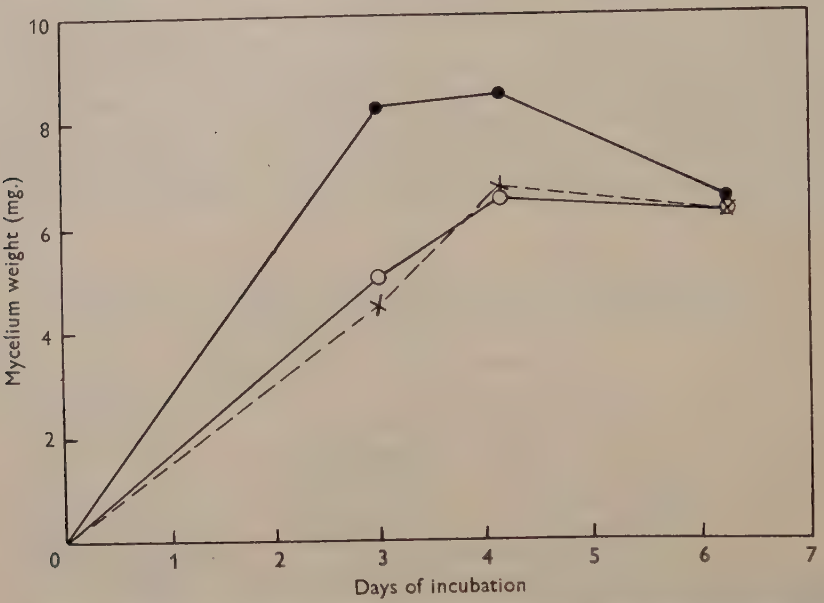

Fig. 1. Effect of calcium and jute extract on the growth of *Chaetomium globosum* strain no. 79. ×---×, on basal medium; ○——○, on basal medium with 6·5 p.p.m. calcium; ●——●, on basal medium with jute extract (concentration as in Table 4).

Effect of glassware

Apart from the evidence reported above, there were other indications that *C. globosum* was very sensitive to minute traces of mineral elements. Erratic results were obtained when flasks other than Pyrex were used; thus, Monax flasks gave abnormal growth in the form of small isolated colonies of the type caused, for example, by traces of Zn or high doses of Ca in the medium. Two Indian-made glasses resulted in different types of abnormal growth and stimulated fruiting. Even Pyrex flasks made in England were somewhat more favourable for fruiting than the American-made flasks used in these experiments. All this suggested that in a purer basal medium, fruiting might in general be further suppressed. However, when strain no. 79 was grown on a medium made with twice-recrystallized salts and with water twice distilled in a Pyrex glass apparatus the same type of mycelium showing immature perithecia was obtained. When the basal medium was made up with KH_2PO_4

Table 6. *Effects of calcium on the growth and fruiting of several species of* Chaetomium

Reference no. of organism	Species	Day of first visible growth		Final growth*		Final fruiting*	
		No Ca	+Ca	No Ca	+Ca	No Ca	+Ca
75	*C. indicum* Corda	5	5	+++	+++	0	+++
108	*C. cancroideum* Tschudy	5	4	+	+++	0	0
109	*C. ochraceum* Tschudy	3	3	+	+++	0	0
110	*C. cochliodes* Palliser	3	3	++	++	0	+++
111	*C. dolichotrichum* Ames	2	1	++	+++	0	+++
112	*C. funicola* Cooke	6	3	+	+++	0	++
115	*Chaetomium* sp.	5	2	+	+++	0	0
116	*C. elatum* Kunze & Schm.	4	4	+	++	0	0

* Increasing number of + signs denotes increasing amounts of growth or fruiting. Zero indicates no fruiting.

instead of K_2HPO_4 the initial pH was 5·0 and growth was rather slow and in the form of small separate colonies, although ultimately a few small but mature perithecia developed. Some evidence was also obtained that on a solid medium (malt agar), artificial light stimulated colony growth of strain no. 79 but retarded the development of perithecia, which also formed abnormal straight hairs. A thicker depth of medium gave more perithecia, within certain limits.

DISCUSSION

Specific instances of stimulation of growth or sporulation of micro-organisms by calcium are meagre in the literature, and there does not seem to be any known instance of the stimulation of the sexual cycle of a fungus by a trace element. Steinberg (1948) found that calcium increased the vegetative growth of several fungi of which *Rhizoctonia solani* showed the most marked response, growth without calcium being only 14·3 % of that with calcium. Discussing the fact that calcium is not known to be required by other fungi, Steinberg has raised the point that with the attainment of greater levels of purity in the basal medium, greater uniformity in the requirement of trace elements by fungi may be found. It is possible that in the experiments here reported some calcium was present as impurity in the basal medium in spite of efforts at purification. Glucose and glassware have been known to contribute calcium (Foster, 1949), and *C. globosum* is clearly sensitive to the kind of glassware; calcium may also have come from the cotton plug or the inoculum. It is therefore possible that the use of a medium more stringently freed from calcium would reveal a calcium requirement for vegetative growth. Some support is lent to this possibility by the fact that calcium stimulates the growth of some other species of *Chaetomium*.

Apart from this reservation, calcium seems to have an effect only on the fruiting mechanism of *C. globosum* as distinct from growth; such instances are at present rare. In the case of fungi, most trace-element work has been confined to species, like *Aspergillus niger*, which do not form the perfect stage, and although the formation of asexual spores is known to be affected in some cases, the effect of trace elements has so far been observed primarily on vegetative growth. Foster & Heiligman (1949) reported that potassium ions (and probably also magnesium and iron) are required for the spore formation of some species of the bacterial genus *Bacillus*, and that the effect is independent of vegetative growth. This is comparable with the action of calcium on *Chaetomium globosum*, although the genetical mechanism of spore formation in the two instances may be very dissimilar.

Inhibition of fruiting, and ultimately of growth, by relatively high concentrations of calcium is comparable with similar effects of growth factors, organic and inorganic, observed by other workers. Calcium is known to be an activator of some enzymes (Foster, 1949); in the case of *C. globosum* it may form part of an enzyme system where it is largely replaceable by strontium or barium. Steinberg (1948) also found calcium partially replaceable by strontium, though not by barium, in the case of *Rhizoctonia solani*. It should be interesting to investigate in what way such an enzyme influences the fruiting of *Chaetomium globosum* without apparently taking part in the purely growth processes, and also to study the nature of the organic factor present in jute extract which, owing to its stimulating action on germination and growth, exerts at least an indirect effect on the fruiting of the species.

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Polysaccharidases of Group A Streptococci

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SUMMARY: Proteinase produced by Group A streptococci was associated with a decrease in hyaluronidase production which was not related to any other known environmental condition. The concentration of proteinase which was correlated with decreased hyaluronidase production was constant for a given strain. Inactive polysaccharidase components in cell-free filtrates were converted to active form by a number of different agents, namely, sodium sulphite, zinc and calcium ions, autoclaved broth and pantothenic acid. Bacterial dextran, pectin and inulin were depolymerized by cell-free filtrates and by partially purified enzymes derived from Type 4, Type 22 and Type 2 strains. 'Depolymerase' was associated with hyaluronidase and amylase in biologically active material prepared by fractional precipitation.

Degradation of starch by hyaluronidase-positive strains of Group A streptococci was described by Crowley (1950), who found that environmental conditions unfavourable for active proteinase production also influenced polysaccharidase activity. The present paper reports further studies of polysaccharidase activity related to the possibility of an inactive precursor analogous to that of proteinase (Elliott & Dole, 1947) and to the hypothesis that depolymerization of viscous substances by Group A streptococci may be the function of one or more enzymes of low specificity.

MATERIAL AND METHODS

Strains. The strains of Group A streptococci were those used by Crowley (1950) in studying amylolytic activity. Type-specific M antigens were identified by the method of Swift, Wilson & Lancefield (1943), and T antigens by slide agglutination (Griffith, 1934).

Cultures. Strains were grown in the same batches of glucose beef infusion broth or in soft agar gels as used for amylase production (Crowley, 1950).

Proteinase. The clotting time of milk + thiolacetate was estimated by the method of Elliott & Dole (1947).

Hyaluronidase. Hyaluronidase was titrated by the M.C.P. test (McClean, 1942). Pooled cadaver synovial fluid kept under toluene was used as substrate. The hyaluronic acid content of the original pool was 1 %, estimated by Dr W. T. J. Morgan (The Lister Institute, Chelsea). This batch was standardized against a cell-free filtrate (strain H713, Type 4) which, after glycerol dialysis, was preserved at −10° and subsequently used to standardize fresh batches of substrate. Synovial fluid formed mucin clot with 2N acetic acid without the addition of extra protein. Horse serum was only added for purposes of comparison with six batches of umbilical hyaluronate prepared in this and other laboratories. No sample of synovial fluid from the pool gave discrepant results like those obtained with different batches of partially purified umbilical hyaluronate.

Amylase. Hydrolysis of 0·2 % starch (AnalaR) was tested by the method of

Crowley (1950), but using 0·5 ml. of substrate instead of 1·0 ml., thus enabling readings to be made at shorter time-intervals. Starches of different grain sizes (wheat, maize, arrowroot, potato, rice and tapioca) were supplied by Messrs Reckitt and Colman, Carrow Works, Norwich, and by British Drug Houses Ltd. Both 0·2 % (w/v) and 1 % (w/v) gels were tested.

Depolymerase. Citrus pectin (Messrs Reckitt and Colman), inulin (Messrs Kerfoot), and bacterial dextran from *Leuconostoc mesenteroides* (East Anglia Chemical Co. Ltd.) were used to test depolymerizing activity. Ethanol, methanol or acetone (AnalaR) were used in preliminary tests to determine the optimal concentrations, pH values and volumes of precipitants suitable to produce a characteristic substrate reaction. The reagents are shown in Table 1, together with physical changes seen by naked eye in substrates undergoing degradation. In all tests 0·5 ml. substrate in sodium β-glycerophosphate (0·126 M) was mixed with 0·5 ml. aqueous dilution of enzyme sample and incubated in a 37° water-bath. The protein in low dilutions of broth interfered with readings in all depolymerase tests, therefore broth dilutions of less than 1/10 were treated with acid-ethanol (95 parts ethanol + 5 parts N-HCl) and after centrifuging to remove precipitate the supernatant fluids were pipetted into acetone, methanol or ethanol at appropriate pH. The lowest dilution of the sample + substrate mixture was tested for reducing sugars or for fructose (furfural test) after incubation, and compared with a broth + substrate control.

Units. Working with twofold dilutions in the defined conditions 0·5 ml. of enzyme dilution in the end-point tube contained 1 unit of enzyme, therefore 1 ml. of the original sample contained 2 units × the reciprocal of the end-point. With starch substrates the unit was equivalent to the Wohlegemuth unit for pancreatic diastase, and with hyaluronate to the M.C.P. unit of Quinn (1948).

Partially purified enzymes. After preliminary precipitation by salt saturation, protein fractions were treated with varied concentrations of ethanol or methanol at −10° and different pH values. Further purification was attempted by fructional precipitation in methanol + water mixtures following the principle of Wittler & Pillemer (1949).

EXPERIMENTAL

Conditions decreasing hyaluronidase production

Cultural conditions. The pattern of hyaluronidase production in a favourable broth was constant for any one strain but showed considerable strain variation. The usual strain pattern was established in Neo-peptone autoclaved broth cultures incubated at 37° for 4–6 days. The combined effects of varying the brand of peptone and the method of sterilization of the buffered beef infusion broth are shown in Fig. 1, together with the period of incubation, and the effect of growth at 23°. This experiment was described when reporting the effect of cultural conditions on amylase production by the same strain, and results for the two enzymes run parallel. Consistent results were obtained with batches of broth made from eight separate lots of beef. Type 22 strains showed similar alterations of hyaluronidase activity.

The results suggested the existence of a heat-labile growth-promoting factor in filtered broth, enhanced by Proteose peptone in cultures grown at 23° because this medium contained the greatest number of viable organisms for the longest time. On the other hand, profuse growth occurred in all the broth cultures, and it seemed possible that there was an enzyme-promoting factor in autoclaved broth which converted inactive precursor to active hyaluronidase or initiated an autocatalytic process analogous to the production of active proteinase (Elliott & Dole, 1947).

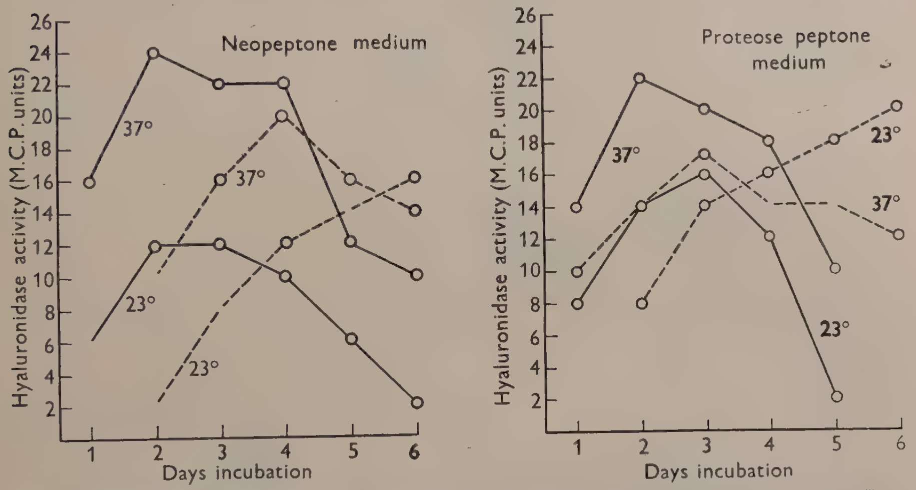

Fig. 1. Changes in hyaluronidase production in varied culture conditions. Strain H713, Type 4 was grown in four different broths, in duplicate cultures incubated at 23° and 37°. Daily samples were frozen, hyaluronidase titrations being made when sets were complete, against the same batch of substrate. ——, autoclaved broth; -----, filtered broth.

Effect of proteinase. Two strains, H713 Type 4 and 63T Type 22, were grown in eight differently constituted soft agar gels. The gels were made up with different amounts of agar and various types of broth (see Fig. 1). In each series, two gels contained bicarbonate buffer, instead of sodium β-glycerophosphate, and one gel contained filtered broth. After 2 days incubation at 37° the cultures were frozen and the liquid thawed out and filtered. High proteinase concentration was correlated with increased agar/broth volume ratio, irrespective of the original broth constituents. The greatest yield of proteinase by one strain was in the medium made up with Neopeptone autoclaved broth most favourable for hyaluronidase production, mixed with 1·5 vol. agar. Log t, the time in minutes taken to clot milk + thiolacetate, was plotted against log c, using twofold dilutions of enzyme sample. Results for a Type 4 strain (Fig. 2) show that a final high proteinase concentration was associated with twofold decrease in hyaluronidase unit value. Results for the Type 22 strain were similar.

Five Type 4 and five Type 22 strains were selected which showed wide variation in hyaluronidase and proteinase production. The ten strains were grown in soft agar containing broth favourable for proteinase production. The hyaluronidase titre of two strains which did not produce proteinase was undiminished. Six strains produced high proteinase concentration, and the usual hyaluronidase titre was halved. Two strongly proteinase-positive Type 4 strains produced no hyaluronidase in these conditions, both strains usually showing low M.C.P. titre. The effect of high proteinase concentration on Type 4 hyaluronidase was greater than the effect on Type 22 hyaluronidase, the proteinase concentration needed to halve the hyaluronidase titre being higher than the corresponding value for Type 4 strains.

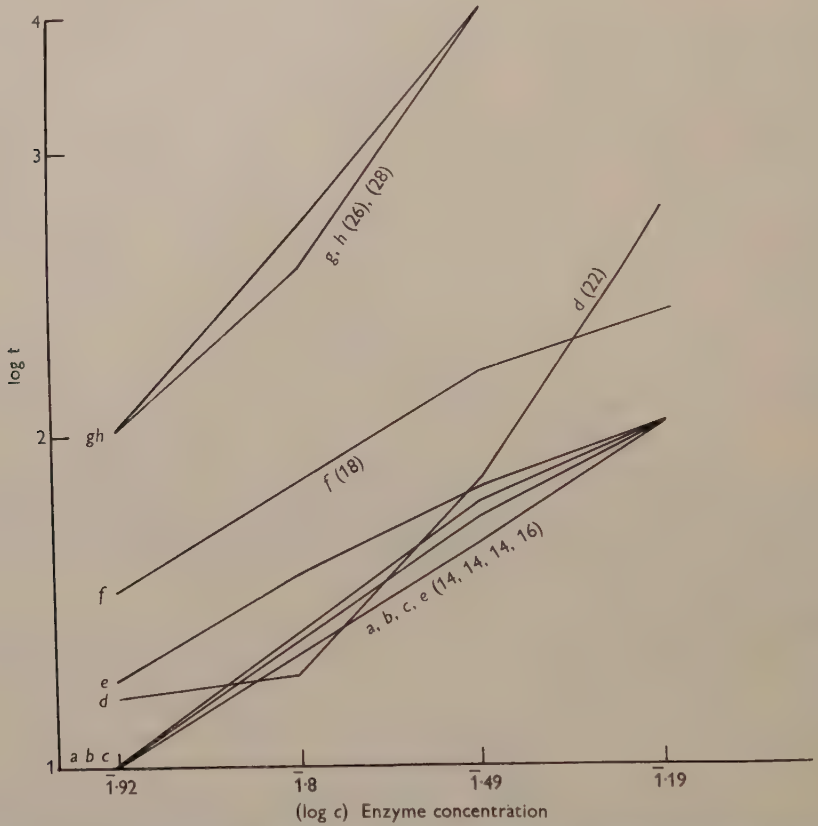

Fig. 2. The hyaluronidase titre of strain H713, Type 4, in different cultures containing different proteinase concentrations. Letters *a*–*h* = different cultures in varied media; numbers in brackets = hyaluronidase titre in M.C.P. units. Log *t* = time in min. taken to clot milk + thiolacetate. The concentration of enzyme in dilutions less than 1/12 was too high to take minute readings.

High proteinase yields were also associated with decreased α-amylase-like activity of hyaluronidase positive Type 4 strains. The β-amylase-like activity of the same strains, mainly catalysing viscosity diminution and partial degradation of starch, was not decreased. The viscosity-decreasing component predominates in Type 22 hyaluronidase (see p. 247). There is at present no evidence to explain the proteinase effect.

Degradation of other polysaccharides

Polysaccharide substrates. The characteristic reactions of control solutions precipitated with ethanol, methanol or acetone are shown in Table 1.

Table 1. *Tests for degradation of polysaccharides*

All solutions were in sodium β-glycerophosphate 0·126 M, pH 7·4.

Incubation 37° water-bath: Dextran 18 hr.; starches 30 min. and 18 hr.; pectin 1 hr.; hyaluronic acid 20 min.; inulin 2 hr. and 18 hr.

Viscosity decrease qualitative test. Rate of filtration through No. 50 Whatman paper as compared with control solution (higher concentration).

Substrates	Concentration in tests	Precipitant or other test	Characteristic reaction	Change in degraded substrate: Chemical	Change in degraded substrate: Physical
Glucose polymers:					
Dextran	3, 1% (v/v)	Methanol 0·3 ml., pH 8·0	Clotted ppt.	Increased turbidity→clear solution	Floccules
Starch	0·5, 0·1% (w/v)	Iodine	Colour change	Colour reading	Floccules, loss of viscosity
Fructose polymer:					
Inulin	1% (w/v)	Acetone 0·5 ml., pH 8·0	Uniform turbidity	Increased turbidity→clear solution	Opalescence fine deposit
Galacturonic acid polymer:					
Pectin	0·5, 0·1% (w/v)	Acetone 1 ml., pH 7·4	Clot	Clot prevention	Loss of viscosity
Mucopolysaccharide:					
Hyaluronic acid	0·2% (v/v)	2 N acetic acid, 0·2 ml.	Clot	Clot prevention, increased turbidity	Loss of viscosity

Physical changes were easier to see in high substrate concentrations, whereas chemical changes were best seen in lower concentrations using minimal volume of precipitant. Buffered pectin solutions formed a clot with acetone at pH 7·4 resembling the mucin-clot of hyaluronic acid + protein. Pectin-clot prevention tests were carried out in the manner of M.C.P. tests. Bacterial dextran was precipitated in irregular clumps by 0·3 ml. methanol at pH 8·0 without turbidity, but a further 0·1 ml. of methanol caused general turbidity. Change in inulin was estimated by increase or decrease in turbidity, together with furfural tests for fructose.

Degradation by culture supernatants, cell-free filtrates and partially purified enzymes. Results with Type 4, Type 22 and Type 2 strains are shown in Table 2. Loss of viscosity was the earliest change in starch and pectin solutions. Opalescence appeared in degraded inulin solutions and coarse floccules in dextran, which became finer after further incubation. Floccules did not appear when the test was carried out at pH value 6·6 but were seen better at pH range 7·4–7·8, than at pH 7·0. All reactions were retarded in the absence of phosphate; the optimal NaCl concentration was not determined. In general the same cultural conditions affected production of depolymerase, amylase and hyaluronidase, but full investigation was not attempted nor have enough representative serological types been tested to form any opinion of the value of screening tests in the estimation of depolymerizing activity by strains other than Types 4, 22 or 2.

Table 2. *Degradation of polysaccharides by extracellular enzymes of Group A streptococci*

All solutions were in sodium β-glycerophosphate buffer (0·126 M), except hyaluronic acid (pooled cadaver synovial fluid). Tests for degradation as in Table 1. Readings ++ = high titres recorded; + = low titres recorded; ± = 1/8 dilution only. Results for partially purified material were based on unit activity/mg. protein nitrogen. Starches: high % amylose (24 %) = soluble, wheat; low % amylose (17 %) = arrowroot, rice, tapioca.

		Substrates					
	Strains	Hyaluronic acid	Starch high % amylose	Starch low % amylose	Bacterial dextran	Citrus pectin	Inulin
Cell free filtrates	H713 Type 4	++	++	++	+	+	++
	63T Type 22	++	·	±*	+	+	+
	4226 Type 2	·	++	+	±	+	±
Partially purified enzymes	Type 4, 5 strains	++	++	++	+	+	++
	Type 22, 3 strains	++	·	+*	++	+	+
	Type 2	·	++	+	+	+	+

* Type 22 enzymes degraded tapioca starch only.

Partially purified enzymes. Fractions precipitated by 25 % methanol at pH 6·4 and 40 % methanol at pH 4·8, and at pH 4·2, all showed hyaluronidase, amylase and non-specific depolymerizing activity. Active material remained in the supernatant after precipitation at pH 4·2, which was finally removed by increasing methanol concentration to 70 %. At first maximum activity was associated with the fraction precipitated at pH 4·2, but after repeated fractionation the main active material became progressively more soluble in methanol, and the best yield was recovered with 80 % methanol which was left to stand at −10° for about 1 week. The available material was insufficient for adequate chemical analysis but appeared to be a water-soluble protein of low molecular weight. The relative activities of Type 4, Type 22 and Type 2 enzymes are indicated in Table 2. Results were reproducible for eleven filtrates, five Type 4 strains, three Type 22 strains, and three filtrates of one Type 2 strain.

Activation of polysaccharidase components

A Type 4 strain, H713, was grown for 2 days in conditions unfavourable for hyaluronidase and amylase production, namely in Neo-peptone filtered broth (1 l.) at 23°. The whole culture was treated with $(NH_4)_2SO_4$ to bring to 0·7 saturation. After standing overnight at 4° the precipitate and cell debris were suspended in distilled water. The suspension was dialysed against running water for 2 days, and against glycerol for 1 day. The extract was free from viable organisms and had about one-fifteenth of the original volume. Graded dilutions in distilled water, sterile tap water or physiological saline were incubated overnight at 37° after various additions. A Type 22 strain, P674, was grown in similar conditions, and material prepared in the same way.

Activation of amylase. Table 3 shows loss of activity after dialysis, restoration of β-amylase activity in salt solutions, and activation of α-amylase after incubation with sodium sulphite enhanced by sodium β-glycerophosphate. The activation of amylase in these conditions was retarded by sodium salicylate. Active amylase-positive concentrates from Type 4 and Type 2 strains were also inactivated in the presence of salicylates in starch hydrolysis tests (Table 4).

Table 3. *Activation of amylase by incubation of an inactive concentrate with sodium sulphite*

Strain H713 (Type 4) was grown for 2 days in filtered broth, the whole culture then precipitated by 0·7 saturation with $(NH_4)_2SO_4$. The precipitate was dialysed against tap water and then against glycerol. This glycerol concentrate was then examined as under. Before precipitation the culture supernatant had no α-amylase activity, and the β-amylase titre was 1/8 after 18 hr. incubation. Iodine test: + + + + = achroic point; + + +, + + = erythrodextrins (α-amylase); +, ± = blue deposit (β-amylase). Recorded after 30 min. and 18 hr. 37°.

	Hydrolysis of 0·2 % soluble starch by final dilution of enzyme sample				
	1/2	1/4	1/8	1/16	1/32
Glycerol concentrate					
+ distilled water	±	·	·	·	·
+ sterile tap water	±	·	·	·	·
+ 0·9 % NaCl	+	±	±	·	·
+ sodium β-glycerophosphate (0·0126 M)	+	+	±	±	·
+ sodium sulphite (0·001 M) and saline	+ + +	+ +	+	±	±
+ sodium sulphite and sodium β-glycerophosphate	+ + + +	+ + + +	+ + +	+ +	+
Boiled glycerol concentrate					
+ sodium sulphite and sodium β-glycerophosphate	·	·	·	·	·

Table 4. *Inactivation of amylase by sodium salicylate*

Results of iodine tests as in previous Tables.
H713 end-point titre of filtrate was 1/64, + + reading, and equivalent to 12 units.

	Hydrolysis of 0·2 % soluble starch, (30 min. at 37°) by final dilution of enzyme sample			
Cell-free filtrates of strains	1/2	1/4	1/8	1/16
H713 (Type 4) dilution in water	+ + + +	+ + + +	+ + + +	+ + + +
H713 (Type 4) + Na salicylate (0·003 M)	±	±	±	·
4226 (Type 2) dilution in water	+ + + +	+ + + +	+ + + +	+ +
4226 (Type 2) + Na salicylate (0·003 M)	±	·	·	·

Hyaluronidase. The hyaluronidase activity of the same fraction was lost after dialysis. The M.C.P. titre was not increased after restoring salts nor after incubation with sodium sulphite. It also showed no increase after incubation with ascorbic acid (0·1–0·01 M), boiled homologous organisms (washed and unwashed) and partially purified homologous hyaluronidase (1 M.C.P. unit). Material prepared from the Type 22 strain was tested in parallel with negative results.

Cell-free filtrates. The α-amylase was activated by sulphite in an inactive cell-free filtrate of the same Type 4 strain, but the hyaluronidase titre was unaltered. Synovial fluid however became turbid, and the highest dilution showing precipitate as compared with the clear control was recorded as end-point. The turbidity titre was increased after incubation with sodium sulphite or sodium thiosulphate (0·08 M), though the hyaluronidase titre was unchanged when measured by mucin-clot prevention. The 'turbidity' titre of a Type 22 filtrate was also increased after incubation with sodium sulphite, but the rise in titre was slight compared with the Type 4 filtrate in which the increase ran parallel to that of α-amylase. Both Type 4 and Type 22 fractions caused flocculation and loss of viscosity in starch solutions after incubation with tap water, but the iodine test remained positive.

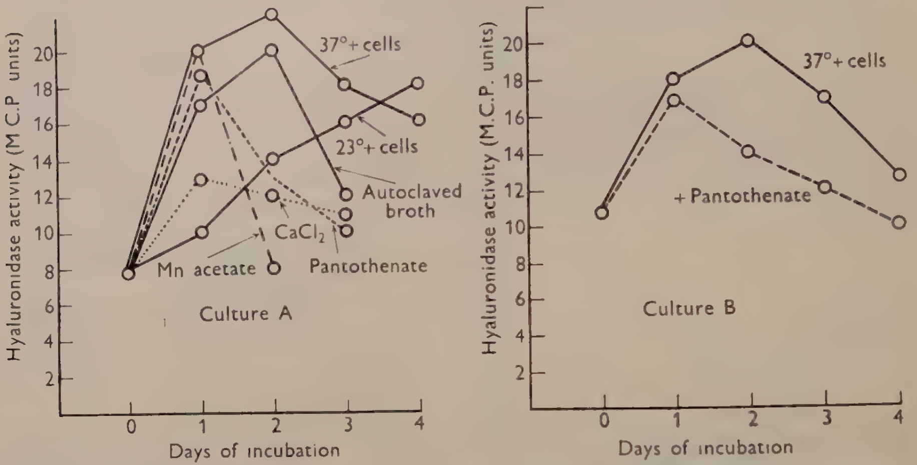

Fig. 3. Effect of pantothenic acid on an inactive cell free filtrate (strain H713, Type 4). Activators were added to graded volumes of filtrate (2–3 day culture at 23°). Neo-peptone autoclaved broth = 3 v/v unconcentrated and v/v concentrate. Hyaluronidase was titrated after reincubation at 37°. (See text for concentrations.)

Traces of calcium, zinc, magnesium, copper and manganese salts were added to inactive filtrates which were incubated with and without sulphite. $ZnSO_4$ (0·0003 M) activated the β-amylase without the addition of sulphite. $CaCl_2$ (0·0025 M) increased α-amylase activity, and gave maximum activity to a dialysed fraction in contrast to NaCl, suggesting that both calcium ions and chloride ions were necessary for activation. Both calcium chloride and manganese acetate (0·00004 M) caused a transient rise in M.C.P. titre (Fig. 3).

Neo-peptone autoclaved broth was frozen and about one-third of the original volume allowed to thaw. This concentrate added in equal volume to an inactive filtrate caused a rise in M.C.P. titre after overnight incubation equivalent to the usual increase of activity when inactive 23° cultures were reincubated at 37° in the presence of cells. The increased activity was only maintained for a few hours, and the filtrate lost all activity after further incubation.

Screening tests with different polymers. The specific requirements for different components were analysed by screening tests with other polysaccharide substrates. The predominant activities were placed in two groups:

depolymerase I, viscosity diminution, degradation of the molecule shown by flocculation, or β-amylase-type activity;

depolymerase II, increase of turbidity, clot prevention, loss of precipitability or α-amylase type activity.

The artificial distinction was made for the purpose of the experiment, but approximates to the grouping made by Meyer (1947) and Rogers (1948). Table 5 shows the effect of different activators on components of depolymerase, comparing the activity of cell-free filtrates against several substrates.

Table 5. *Activation of depolymerase: estimation of components by screening tests with several substrates*

+, ± = greater or lesser activity; V = loss of viscosity without changed iodine reaction; I = physical change, flocculant precipitate, opalescence or turbidity; II = clot-prevention, turbidity changes with alcohol or acetone, or loss of iodine colour.

	Substrates							
	Starch 0·2 %		Dextran 2 %		Pectin 0·2 %		Inulin 2 %	
Weak or inactive culture filtrates	I	II	I	II	I	II	I	II
Strain H713, Type 4								
Filtrate + tap water	±	·	±	·	±	·	·	·
+ $ZnSO_4$ (0·0003 M)	+	·	±	·	±	·	·	·
+ $CaCl_2$ (0·0025 M)	+	+	±	±	+	+	·	+
+ Na_2SO_3 (0·001 M)	+	+	±	±	+	+	·	+
+ 3 vol. Neo-peptone autoclaved broth, or 1 vol. concentrate	+	+	±	±	+	+	·	+
Strain 63T, Type 22								
Filtrate + tap water	V	·	+	·	+	·	·	·
+ $ZnSO_4$ (0·0003 M)	V	·	+	·	+	·	·	·
+ $CaCl_2$ (0·0025 M)	·	·	+	+	+	+	·	±
+ Na_2SO_3 (0·001 M)	·	·	+	+	+	+	·	±
+ 3 vol. Neo-peptone autoclaved broth or 1 vol. concentrate	·	·	+	+	+	+	·	±

The addition of autoclaved Neo-peptone broth increased the activity of all components, lending support to the hypothesis of an enzyme-promoting factor made available by heating broth. Dole (1946), studying the effect of peptone on the growth of streptococci, reported that Neo-peptone (DIFCO) had a high calcium content. This suggested that the enzyme-promoting factor in the broth might be enhanced by calcium, explaining the superiority of Neo-peptone compared with Proteose peptone in increasing hyaluronidase production.

The effect of pantothenic acid. Growth-promoting activity for *Escherichia coli* has been attributed to pantothenic acid conjugate in muscle extracts (King & Cheldelin, 1950). Pantothenic acid (DL, calcium salt) was added to inactive filtrates (2 mm. loopful 0·02% (w/v) to 10 ml. broth); the M.C.P. titre had increased after overnight incubation at 37°. The rise in titre was less than that after adding autoclaved Neo-peptone broth but greater than after adding $CaCl_2$ (0·0025 M). An autoclaved pantothenate solution was less active. Activity after addition of β-alanine and γ-butyrolactone was not fully investigated. Fractions obtained by alcohol precipitation of filtered broth have so far failed to promote growth, and fractions of autoclaved broth, to promote enzyme production. There is no evidence at present to explain the effect of pantothenic acid.

DISCUSSION

The depressant effect of proteinase in high concentration on hyaluronidase may explain the variable activity of some Group A strains recorded by Meyer (1947). Proteinase activity by strains of the T antigen groups 4, 24, 26, 28, 29 and 46 was associated with absence of polysaccharidase production (Crowley, 1950). Of Type 22 strains 90% had both proteinase and hyaluronidase activity, and the impression that different components predominated in the Type 22 enzyme was confirmed by screening tests against other polysaccharide substrates. The components designated depolymerase I, which catalyse viscosity diminution and degradation of the molecule, resemble phosphatases in activity. Since cell-free filtrates degraded five polymers it seems justifiable to conclude that the enzyme is a phosphatase of low specificity as suggested by Robertson, Ropes & Bauer (1940). The activity of the second group of components resembled both hyaluronidase (estimated by turbidity change) and α-amylase.

Sodium sulphite, which converted inactive precursor to amylase, also activates streptolysin S (Herbert & Todd, 1944). The inactivation of amylase by salicylates raises several problems. Heparin inactivates both hyaluronidase (McClean, 1942) and amylase (Crowley, 1950). Calcium ions activate amylase and the anticoagulant effect of heparin has been attributed (among other factors) to the binding of calcium. The tentative identification of α-amylase with one or more components of Type 4 hyaluronidase suggests that it may be premature to abandon the theory of anti-hyaluronidase activity by salicylates (Guerra, 1946), which Swyer (1948) believed to be due to contamination with histamine or a similar substance.

Though the role of pantothenic acid conjugate, of the free acid or its cleavage products in growth and enzyme promotion is not clear, it may be worth noting that Type 4 strains which lost polysaccharidase activity after serial mouse-passage also lost ability to utilize high concentrations of yeast nucleic acid. It has been suggested that pantothenic acid is involved in purine metabolism besides being an essential growth factor for organisms which ferment carbohydrates (Baldwin, 1948).

In conclusion the non-specific depolymerizing activity of strains which degrade several polysaccharides was termed depolymerase to describe imperfectly defined activities in test conditions which were not necessarily optimal.

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The Virulence of *Haemophilus pertussis* for Mice by the Intranasal Route

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With a note by J. O. Irwin on the relation between LD 50 and average time to death in 25 strains of *H. pertussis*

SUMMARY: Freshly isolated strains of *Haemophilus pertussis* vary considerably in their virulence for mice when tested by the intranasal route; this variation is continuous and must be regarded as a quantitative not a qualitative character. In order to titrate virulence it is necessary to achieve and maintain a standard technique with cultures and to use 'standard' mice kept under standardized conditions. It was not possible to show a correlation between virulence (as measured by the LD50) and either the viable count or the presence of toxin or haemagglutinin in the suspensions used in the virulence test. Nor was it possible to show any correlation between virulence and the 'age' of the culture, either in number of subcultures, time elapsing between swabbing and testing, type of swab or duration of illness (up to three weeks) when the swab was taken. Some strains kill mice more quickly than others; this ability is attributed to a 'quick-killing factor'.

The experiments reported here were designed to titrate the virulence of *Haemophilus pertussis* by the intranasal technique of Burnet & Timmins (1937) in order to show whether freshly isolated strains of this organism from different cases or from different epidemics varied in virulence as strains of other organisms appear to. A survey of the literature shows that considerable differences in the virulence of different strains have been reported, though details of the strains are seldom given. It was also hoped to correlate the variations in virulence which were found with certain other factors which it was felt might be involved. First, certain details of method are stressed, since it soon became obvious that in work of this sort it was more important to develop a standarized technique, which was thereafter rigidly adhered to, than to change the technique constantly by the introduction of improvements.

MATERIAL AND METHODS

Collection of strains. Strains of *H. pertussis* examined were freshly isolated from patients with whooping-cough in Colindale, Leeds, Manchester and Oxford. The first subculture from the original cough- or swab-plate was sent to this laboratory. All strains reported in this paper were in Phase I of Leslie & Gardner (1931) in that they agglutinated to titre with Phase I serum and would only grow on Bordet-Gengou medium.

No attempt was made to isolate cultures from single colonies; in most cases a heavy inoculum from confluent growth was used. When separate colonies only were available at least twelve colonies were picked off with a loop for a single subculture.

Method of test

Preparation of bacterial suspensions. Each strain was grown on Bordet-Gengou medium for 20 hr., the growth scraped off into 1 % Bacto-casamino acids (Baird & Tatlock, London) and standardized by opacity against Brown's Opacity Tubes (Burroughs Wellcome and Co. Ltd., London) assuming that *H. pertussis* was equivalent to *H. pfeiffer.* Four dilutions were used containing 2500, 250, 25 and 2·5 million organisms/ml., as the end-point usually lay within this range. Not more than 2 hr. was allowed to elapse between the dilution of the culture and the last instillation of the mice.

The importance of the age of the culture has been stressed by Gray (1946) and this has been confirmed (see Table 1).

Table 1. *Virulence of an* Haemophilus pertussis *strain* (*C*4039, *second subculture*) *after different periods of incubation*

Period of incubation (hr.)	LD 50 (millions)	Haemagglutinin titre
20	0·58	24
42	6·5	192
70	31·0	394

Mice. All mice used came from the same closely bred strain. For each test 40 mice, all of one sex and weighing 14–18 g., were distributed at random into four boxes, each dose being tested in 10 mice. They were fed on Rowett Research Institute pellets (North-Eastern Agricultural Co-operative Society, Aberdeen) for the whole of the experiment and were given water *ad lib.* The importance of using the same strain of mice is shown in Table 2, which compares the virulence of the same subculture of an *H. pertussis* strain tested in three batches of mice from different sources. Whereas mice from sources S1 and C are about the same, mice from source S2 differ very considerably in their susceptibility to pertussis infection.

Table 2. *Comparison of virulence of* Haemophilus pertussis, *strain no.* 323, *in mice from three different sources*

	LD 50 (on 21st day)	
Strain of mice	Test 1	Test 2
S1	40·0	80·0
C1	33·0	60·0
S2	170·0	200·0

Culotta, Martin & Liebow (1938) drew attention to the importance of weight or size of mice in experimental infection with *H. pertussis*; with a standard dose and using the same strain they found 93 % mortality in young mice weighing 10–12 g. and only 66 % mortality in adult mice weighing 20–25 g. This has been confirmed, and Table 3 shows the importance of the weight of the mice used in work of this kind.

Table 3. *Comparison of the LD50 of the same strains tested simultaneously in mice of different weights*

Strain of *H. pertussis*	Group	Average weight of mice at start of experiment (g.)	LD 50 (millions)
C10467	A	14·2	0·67
	B	21·3	2·1
	C	27·0	56·0
C247649	A	15·5	0·66
	B	24·0	8·7

Procedure. The mice were anaesthetized with an ether-chloroform mixture and a dose of 2 drops of each dilution from a pipette, calibrated to deliver 50 drops/ml., was given to each mouse (i.e. doses of 100, 10, 1 and 0·1 million organisms). Only those mice which absorbed the dose quickly and completely were included in the test; any mouse which sneezed, blew or swallowed the dose or part of the dose was excluded. Mice which swallow some of the dose are sometimes difficult to detect and may account for some of the unexpected survivors. The mice were watched in the early experiments for 56 days and in the later experiments for 28 days. Mice dying within 24 hr. were considered 'accidents' and excluded.

Table 4 shows four strains of high, medium and low potency.

Statistical treatment of results. The virulence of different strains was measured by the LD50, calculated by the method of Reed & Muench (1938) from the figures obtained from the four dose levels actually used.

In the first series of twenty-five strains the LD50 was determined for each week up to 8 weeks (Table 5). Although the LD50 of some strains continued to fall with the passage of time, there was little change in the order of virulence of the strains. It was decided that 4 weeks was a reasonable period to keep the mice and that the virulence of a strain should be measured by the LD50 calculated by the Reed & Muench method on the 28th day. For convenience, strains were arranged in five arbitrarily chosen groups, as follows: group A, LD50, $<1\times10^6$ organisms; group B, LD50, 1×10^6–5×10^6 organisms; group C, LD50, 5×10^6–25×10^6 organisms; group D, LD50, 25×10^6–125×10^6 organisms; group E, LD50, $>125\times10^6$ organisms.

Reproducibility of results. It is difficult in this type of testing to reproduce the exact condition of a test. On several occasions tests were set up in parallel to show whether comparative results could be obtained (Table 6). These tests probably show a degree of accuracy and reproducibility not always achieved.

RESULTS

When the first series of strains of *H. pertussis* was tested, mice were not always immediately available, and certain of the cultures of *H. pertussis* which were contaminated, had to be plated once or twice to purify them. The great variation in virulence shown by these strains (Table 5) suggested that they might be undergoing some form of degeneration, although they remained in Phase I by agglutination. A second series was therefore tested in which only those challenge suspensions which could be prepared from the 2nd or 3rd subculture from the cough-plate were used. The same variations occurred in this series. Details of the day on which the swabs were taken, etc., were obtained for each strain, and it was found that although the strains were tested as soon as possible after being received at Elstree, nevertheless some days had often elapsed between swabbing and the mouse test. For instance, strain C978 was received 12 days after swabbing, and a suitable subculture for infecting mice could not be prepared until 3 days later—a delay of 15 days.

Table 4. *Details of intranasal virulence test on* Haemophilus pertussis *strains of high, medium and low virulence*

Strain of *H. pertussis*	Dose (no. of cells × 10⁶)	Day of death of ten mice									
G928	100	2	2	2	2	2	2	3	3	6	6
	10	3	3	3	8	9	9	10	11	14	17
	1	10	19	37	43	S	S	S	S	S	S
	0·1	23	26	S	S	S	S	S	S	S	S
G1041	100	6	6	6	6	6	7	9	12	13	*a*
	10	12	14	18	20	23	24	28	32	S	*a*
	1	31	33	41	43	46	53	S	S	S	S
	0·1	21	29	50	50	S	S	S	S	S	S
G942	100	2	9	9	10	13	14	15	15	18	S
	10	10	10	21	S	S	S	S	S	S	S
	1	8	32	S	S	S	S	S	S	S	S
	0·1	26	S	S	S	S	S	S	S	S	S
M5	100	11	27	27	S	S	S	S	S	S	S
	10	S	S	S	S	S	S	S	S	S	S
	1	15	S	S	S	S	S	S	S	S	S
	0·1	54	54	S	S	S	S	S	S	S	S

S = survivor; *a* = mouse lost by accident.

	Weeks							
	1	2	3	4	5	6	7	8
	LD 50 (millions)							
G928	32	5·5	4·1	3·1	3·1	2·1	2·1	1·0
G1041	83	38	10	7	4	3	0·9	0·6
G942	180	62	23	17	10	10	10	10
M5	194	170	160	122	122	122	122	108

As it was possible that some form of degeneration might occur in this period, an arrangement was made with the Central Public Health Laboratory, Colindale, to collect special cough-plates, to subculture at once and to use this subculture to infect mice at the earliest possible time after collection of the cough-plate from the patient. Seven strains were collected and used. These cultures showed considerable variation in virulence (Table 7). It is therefore assumed that these variations reflect inherent characters of *H. pertussis* strains. At present we do not know enough to explain these variations in mouse virulence of strains which have all been isolated from patients with clinical whooping-cough.

Table 5. *LD50 of the first series of twenty-five strains of* Haemophilus pertussis *tested for virulence*

Calculated each week for 8 weeks.

	Week							
	1	2	3	4	5	6	7	8
Strain of *H. pertussis*	LD 50 (millions)							
863	24	3	1	0·7	0·7	0·7	0·7	0·6
888	8	4	2	1	1	1	1	1
894	55	9	9	9	9	9	9	8
928	32	6	4	3	3	2	2	1
941	33	8	7	6	6	6	6	6
942	180	62	23	17	10	10	10	10
M1	137	70	55	55	46	46	46	46
M2	174	174	174	152	128	100	90	90
907	167	127	100	90	90	90	89	68
910	127	108	100	75	65	58	25	25
962	155	77	77	77	62	56	10	3
978	132	65	56	17	17	7	7	7
M5	194	170	160	122	122	122	122	108
M6	170	75	54	40	40	31	15	15
L2	140	68	60	40	5	0·8	0·8	0·8
947	166	143	143	143	143	123	93	81
959	120	73	68	55	55	55	47	47
983	200	180	140	100	75	45	5	4
992	182	141	109	42	36	29	23	23
993	166	150	116	100	81	58	49	39
1016	151	10	3	0·9	0·6	0·5	0·4	0·4
1030	68	45	9	6	4	4	0·7	0·4
1031	91	82	0·7	0·6	0·6	0·5	0·5	0·5
1041	83	38	10	7	4	3	0·9	0·6
L3	184	184	135	86	73	73	52	40

Table 6. *Repeated virulence tests to show reproducibility of results*

Strain of *H. pertussis*	No. of subculture	Date of test	LD50 (millions), 28th day
C407	3	19. vii. 48	0·24
	3	19. vii. 48	0·28
	3	19. vii. 48	0·33
C7386	3	13. viii. 48	26·0
	3	13. viii. 48	28·0
	3	13. viii. 48	29·3
C10487	2	9. iv. 49	0·67
	2	9. iv. 49	0·65
407P	3	19. vii. 48	0·33
	4	20. vii. 48	0·28
	5	21. vii. 48	0·37

The distribution of strains in order of virulence in the various tests is shown in Table 8.

Table 7. *LD 50 of strains of* Haemophilus pertussis (*2nd subculture*) *tested in mice within 48 hr. of taking the swab*

Strain of *H. pertussis*	LD 50 (millions)	Quick-killing factor
C 2843	35·0	−
C 2476	0·66	+
C 4039	0·58	+
C 4491	30·0	+
C 6403	51·0	−
C 7383	3·75	−
C 10487	0·67	+

Table 8. *Classification of strains of* H. pertussis *according to LD 50*

Series ...		1	2	3	4	Total*
Subculture tested ...		2–22	2 or 3	2	2–8	2–22
Group	LD 50 (millions)					
A	< 1·0	3	10	3	15	42
B	1·0 and < 5·0	2	6	1	10	19
C	5·0 and < 25·0	6	9	0	7	22
D	25·0 and < 125·0	12	10	3	10	35
E	> 125·0	2	1	0	0	3
	Total	25	36	7	42	121

* All strains tested including replicates, etc.

Quick-killing factor (*q.k.f.*)

Another phenomenon was observed: the method of titrating virulence by the LD 50 depends on the total number of mice killed at the different dose levels and ignores the time taken to kill; when, however, time to death is taken into account it is seen that two strains of equal LD 50 may differ considerably (Table 9).

The difference is most obvious at the 100×10^6 dose level, where the average time of death was 2·0 days for strain L 33 and 8·9 days for strain L 9, though the LD 50 for these strains was 0·55 and 0·5 respectively; similarly, in strain G 941 the average time of death was 4·7 days and in strain L 46, 13·1 days.

The presence or absence of the quick-killing factor was indicated by the number of mice killed in 3 days at the 100×10^6 dose level; if more than half the mice were killed the strain was considered positive for q.k.f.; if less than half, negative. The distinction between the two kinds of strain is usually quite clear and is usually reflected at the 10×10^6 dose level (Table 10).

Little more was discovered about the q.k.f. except that it could be lost when the strain was subcultured too long and recovered by mouse passage up to a point, after which it was permanently lost. The time at which this loss took place differed with the strain. Some preliminary experiments indicated that the q.k.f. is probably not antigenic, as demonstrated by the usual methods. Vaccines prepared from q.k.f. positive strains are not markedly superior to vaccines prepared from q.k.f. negative strains, when tested by Kendrick's method (Kendrick *et al.*, 1947).

Table 9. *Variation in time of death of mice caused by different strains of* Haemophilus pertussis *at the same dose level*

Strain of *H. pertussis*	Dose (millions)	Period to death in ten mice (days)										Average time of death (days)	LD 50 (28th day) (millions)
L 33	100	2	2	2	2	2	2	2	2	2	2	2	0·55
	10	2	2	2	4	6	19	20	21	S	S	8·5	
	1	11	13	14	16	16	21	S	S	S	S	15·1	
	0·1	13	15	19	20	20	20	S	S	S	S	17·8	
L 9	100	4	7	7	8	9	9	10	10	12	13	8·9	0·5
	10	6	11	13	13	14	16	24	S	S	*a*	13·8	
	1	13	14	16	17	18	18	20	22	28	S	18·4	
	0·1	18	22	28	28	28	S	S	S	S	S	24·8	
G 941	100	2	2	2	2	2	2	2	3	7	23	4·7	6·4
	10	5	6	6	9	9	9	11	S	S	S	7·8	
	1	5	21	S	S	S	S	S	S	S	S	13·0	
	0·1	S	S	S	S	S	S	S	S	S	S	—	
L 46	100	6	7	7	9	11	17	18	20	23	S	13·1	8·3
	10	10	12	13	15	17	20	S	S	S	S	14·5	
	1	5	S	S	S	S	S	S	S	S	S	—	
	0·1	23	S	S	S	S	S	S	S	S	S	—	
L 14	100	2	2	2	3	7	S	S	S	S	S	3·2	36·0
	10	4	5	7	18	19	S	S	S	S	S	10·6	
	1	22	S	S	S	S	S	S	S	S	S	—	
	0·1	9	S	S	S	S	S	S	S	S	S	—	
C 5467	100	4	9	9	12	15	17	17	21	S	S	13·0	35·0
	10	15	20	21	26	S	S	S	S	S	S	20·5	
	1	S	S	S	S	S	S	S	S	S	S	—	
	0·1	S	S	S	S	S	S	S	S	S	S	—	

S = survivor: *a* = mouse lost by accident.

Table 10. *Strains of* Haemophilus pertussis *tested for quick-killing factor, classified according to virulence*

Quick-killing factor: ...		+	−	+	−	+	−
Group	LD 50 (millions)	All-strains		2nd–3rd subcultures		Special strains*	
A	< 1·0	20	11	4	6	3	0
B	1·0 and < 5·0	8	8	2	4	0	1
C	5·0 and < 25·0	6	8	4	5	0	0
D	25·0 and < 125·0	1	15	1	8	1	2
E	> 125·0	0	2	0	1	0	0

* See Table 7.

Correlation of mouse virulence with other factors

The following factors were tested for correlation with mouse virulence (LD 50) or with the q.k.f.: (*a*) viable count; (*b*) toxin content; (*c*) haemagglutinin of Keogh & North (1948); (*d*) 'age' of culture in terms of number of daily subcultures; (*e*) 'age' of culture in terms of time between swab being taken and challenge; (*f*) duration of illness when swab was taken; (*g*) type of swab, prenasal or postnasal.

Since suspensions used in virulence tests are made up by opacity (total count) and since it is probable that the ratio total count: viable count varies from strain to strain and from suspension to suspension of one strain, it is possible that suspensions which contain the highest proportion of viable organisms might be the most virulent. Toxin was an obvious factor to be investigated in relation to the q.k.f., because the time and type of death suggest toxin, from its known characters. Keogh & North (1948) have shown that their haemagglutinin is antigenic and they suggest that it is intimately connected with virulence. Its nature and function in relation to virulence, etc. are not yet clearly understood. Gray (1946) pointed out the importance of the 'physiological age' of a culture and it may be that the 'age' of the culture in terms of the number of subcultures from the original plate is important, though this might affect the LD 50 and q.k.f. independently, particularly in cultures which degenerate rapidly. It has been suggested by Evans (1949) that the kind of organism isolated from prenasal swabs differs in growth and morphology from organisms isolated from postnasal swabs or cough-plates. If this is so, they might also differ in virulence. Toomey, Ranta & Takacs (1935) showed that a less virulent form of organism may be found in the throats of patients in the later stages of the disease. It was not possible to test all strains for each of the above factors; only a representative sample of strains could be tested for each.

(*a*) *Viable count and virulence*

Certain cultures were counted by the method of Miles & Misra (1938). As will be seen in Table 11, there is apparently no correlation between the viable count and the LD 50, nor between the viable count and the q.k.f. By chance, most of the strains tested in this group were of high virulence and only two strains were q.k.f. negative.

Table 11. *Comparison of virulence* (*LD* 50) *and viable count*

Strain of *H. pertussis*	Count (millions)		Ratio, total: viable	LD 50 (millions) calculated on	
	Total	Viable		Total count	Viable count
G1127	100	108	0·9	0·2	0·22
L16	100	88	1·14	4·1	3·61
L14	100	75	1·33	36·0	27·0
G1127–3	100	72	1·39	0·1	0·07
L15	100	71	1·41	7·5	5·3
C4576	100	56	1·78	0·4	0·22
L20	100	40	2·5	2·7	1·08
L21	100	40	2·5	9·4	3·76
G863–3	100	34	2·94	0·55	0·19
L22	100	20	5·0	67·0	13·4
G863–2	100	18	5·56	1·0	0·18

(*b*) *Toxin and virulence*

Five strains were tested for toxin by the intravenous route in mice at the same time that they were tested for virulence by the standard method (Table 12). Though the LD 50 varied considerably there was little difference in the toxin titre; there was also no correlation between toxin and q.k.f. This result bears out the findings of Cruickshank & Freeman (1937) who tested twenty-one strains by the intraperitoneal route, and found that the M.L.D. was remarkably constant in seventeen strains, being 6000×10^6–9000×10^6. We now know that the intraperitoneal route as used by Cruickshank measures toxin.

Table 12. *Comparison of virulence* (*LD* 50) *and toxigenicity in five strains of* Haemophilus pertussis (*2nd subculture*)

Strain of *H. pertussis*	Virulence LD 50 (millions)	Toxin, LD 50 (millions × 10^3)	Quick-killing factor
C4576	0·4	5·4	+
L13	3·6	2·0	−
C5146	6·2	3·6	+
C5467	35·0	4·8	−
L14	36·0	3·6	+

In another experiment toxin titrations were carried out in rabbits with similar results; there was no correlation between the toxicity of the strain as measured intradermally in rabbits and the virulence or the q.k.f. titrated intranasally in mice.

(*c*) *Haemagglutinin and virulence*

As Keogh & North (1948) have suggested that their haemagglutinating factor is intimately connected with virulence, the haemagglutination titre was tested in a number of strains when they were tested for virulence.

Method. Suspensions for the haemagglutination test were prepared in saline or Casamino acid and standardized to contain $10{,}000 \times 10^6$ organisms/ml., and serial twofold dilutions were prepared from these suspensions.

Red blood cells were prepared by centrifuging 1 ml. of fowl's blood, washing the red blood cells (r.b.c.) three times in saline and re-suspending in 50 ml. of saline. This 'strong' suspension can be kept 3 or 4 days in the refrigerator; before use it is diluted 1/4 with 0·85 % saline. It was found that 0·5 ml. of this dilute suspension when added to 0·25 ml. of bacterial suspension gave the optimum amount of red cells. The exact quantity of red cells did not affect the titre as long as there were not too few cells to give a clear positive and not too many to mask a weak positive.

For the test 0·25 ml. of the dilution of bacterial suspension was placed in a 12 mm. × 55 mm. tube and 0·5 ml. of r.b.c. suspension added. The racks were shaken and incubated at 37° for 1 hr. The tubes were then shaken and put in the refrigerator to allow the cells to settle before the test was read; shaking after incubation and settling in the refrigerator gave a more distinct end-point. The test could be read at any time after 2–30 hr. in the refrigerator without any modification of the end-point. The end-point is taken as the reciprocal of the final dilution of bacterial suspension.

It was not possible to estimate haemagglutinin on all strains. Strains therefore fall into two groups: (1) strains in which the suspension used for the virulence test was tested for haemagglutination; (2) strains in which other subcultures were tested for haemagglutinin. With some strains, subcultures in both groups were tested.

The virulence test suspensions in §1 of Table 13 were grown for 20 hr. on Bordet-Gengou media in all cases. In §2 the average titres shown were from subcultures grown either on solid or liquid media from 24 hr. to 5 days.

Table 13. *Numbers of strains of* Haemophilus pertussis *tested for haemagglutinin classified according to mouse virulence* (*LD* 50)

§1	Haemagglutinin titres of virulence test suspensions					
LD 50 (millions)	192–96	48–24	12	±12–±6	0	Totals
<1·0	2	4	3	2	3	14
1·0 and <5·0	.	6	1	.	4	11
5·0 and <25·0	1	4	2	1	2	10
25·0 and <125·0	1	3	3	.	2	9
>125·0	.	1	.	.	.	1
Totals	4	18	9	3	11	45

§2	Average haemagglutinin titres of suspensions other than virulence test suspensions					
LD 50 (millions)	192–96	48–24	12	±12–±6	0	Totals
<1·0	5	5	2	.	7	19
1·0 and <5·0	1	5	.	2	1	9
5·0 and <25·0	7	5	.	1	2	15
25·0 and <125·0	5	3	4	.	1	13
>125	.	1	.	.	.	1
Totals	18	19	6	3	11	57

All Phase I strains may produce haemagglutinin under suitable conditions; of the sixty-eight strains tested, eleven never produced haemagglutinin and the remaining fifty-seven strains produced it fairly regularly. There were a few occasions on which haemagglutinin was not produced when expected, but this was possibly due to faulty media. Of the eleven virulence test suspensions which were negative for haemagglutinin, the stock strains of seven were positive or trace when tested on other occasions, and four were always negative. Of the eleven negative strains in §2, one strain was positive in two different suspensions and negative in ten other suspensions, the other ten strains were invariably negative; their LD 50 varied from 0·1 to 86·0 million.

Table 14 shows in greater detail the characteristics of some typical strains from each section. It will be seen that there is little correlation between the haemagglutinin titre and either the LD 50 or the q.k.f. It must be borne in mind that the titre of haemagglutinin *in vitro* may not be a measure of the activity (if any) of this factor *in vivo*.

(*d*) *Age of culture when tested, in terms of number of subcultures, and virulence*

The age of the culture, in terms of the number of subcultures between the original swab plate culture and the actual culture used for the mouse challenge, was investigated, since there is evidence that cultures decline in virulence on subculture in the laboratory and may do so at different rates. Of the thirteen strains tested on more than one occasion, seven remained stable for virulence over the period of test, and six proved to be unstable.

Table 14. *Comparison of haemagglutinin and virulence (LD 50) in a number of strains of* Haemophilus pertussis

Strain of *H. pertussis*	No. of subcultures	Haem-agglutinin titre of mouse challenge suspension	LD 50	Quick-killing factor	Average haemagglutinin production of other suspensions
G 863	6	192	0·66	−	+ + +
G 962	5	192	77·0	−	NT
L 11	3	96	0·75	+	+ +
L 45	22	96	9·4	−	NT
L 12	3	48	0·1	+	+ + +
L 46	22	48	8·67	−	NT
G 894	—	48	9·3	+	+ +
L 14	2	48	36·0	+	+ + +
G 959	7	48	55·0	+	+ + +
L 54	9	24	1·7	+	NT
L 16	2	24	4·1	+	+ + +
C 5146	2	24	6·2	+	+ +
G 992	4	24	42·0	−	+
G 947	6	24	143·0	−	+ +
L 45	5	12	0·64	+	NT
L 15	2	12	7·5	+	+ + +
G 978	2	12	17·0	−	±
L 2	2	12	40·0	−	+
G 983	4	12	100·0	−	±
C 4576	2	±12	0·4	+	+ +
L 49	10	±12	0·84	+	NT
G 1101	3	0	0·55	−	0
G 1144	2	0	0·75	−	0
C 3627	2	0	3·8	−	0
L 7	4	0	10·0	−	0
L 17	2	0	0·7	−	+ +
L 10	3	0	1·0	−	±
L 20	3	0	2·7	+	+ +
G 894	2	0	8·0	−	+ + +
L 21	3	0	9·4	+	+ +
L 18	2	0	56·0	−	+ +
G 1096	3	NT	0·1	+	0
C 15S	3	NT	0·46	−	0
G 1016	2	NT	0·85	−	0
G 1030	2	NT	6·4	−	0
L 3	2	NT	86·0	−	0

Haemagglutinin production: + + +, 96 or 192 or more; + +, 24 or 48; +, 12; ±, ±12 or ±6; 0, ±3 or 0. NT, not tested.

In Table 15 details of a selection of strains of the two types are shown. The degeneration in respect to this one characteristic is obvious in the two 'unstable' strains. Of the 'stable' strains L 58 remained constant for virulence for 132 daily subcultures, and L 57 for seventy-two. Strain L 49 remained reason-

Table 15. *Virulence of cultures of* Haemophilus pertussis *subcultured daily on to fresh Bordet-Gengou medium*

Strain of *H. pertussis*	LD 50 (millions) after varying numbers of subcultures shown in brackets			
L45	0·64 (5)	9·4 (22)	34·0 (48)	65·0 (58)
L46	0·67 (5)	8·3 (22)	66·0 (39)	.
L49	0·8 (5)	0·84 (10)	3·0 (62)	26·0 (161)
L57	0·17 (2)	0·84 (72)	.	.
L58	9·3 (6)	10·0 (132)	.	.

ably virulent for about sixty-two subcultures, but when next tested, at the 161st subculture, showed some degeneration.

There is no evidence that LD 50 or q.k.f. depends simply on the number of times the strain has been subcultured.

Table 16. *Classification of strains of* Haemophilus pertussis *according to virulence* (*LD* 50) *and number of subcultures tested*

		No. of times subcultured																
		2	3	4	5	6	7	8	9	10	12	14	15	22	32	34	35	
Group	LD 50 (millions)	No. of strains in the various groups																Totals
(*a*) Forty-five strains subcultured at daily intervals																		
A	<1	4	3	2	1	.	.	.	1	.	1	.	.	.	1	1	1	15
B	1 and <5	3	3	1	.	.	.	1	2	.	.	.	1	.	.	.	.	11
C	5 and <25	7	1	2	.	.	.	.	.	.	.	.	1	.	.	.	.	11
D	25 and <125	4	1	.	.	.	.	.	.	.	1	2	.	.	.	.	.	8
E	>125	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Totals		18	8	5	1	0	0	1	3	0	2	2	2	0	1	1	1	45
(*b*) Forty-eight strains subcultured irregularly or at more than daily intervals																		
A	<1	3	8	2	2	2	.	2	.	1	1	.	1	.	.	.	.	22
B	1 and <5	2	1	1	.	.	.	.	1	.	.	.	.	.	.	.	.	5
C	5 and <25	2	5	2	.	1	.	.	.	.	.	.	.	2	.	.	.	12
D	25 and <125	3	.	3	1	.	1	.	.	.	.	.	.	.	.	.	.	8
E	>125	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	1
Totals		10	14	8	3	4	1	2	1	1	1	0	1	2	0	0	0	48

Table 16 shows the numbers of strains tested at each subculture arranged in the five groups according to the LD 50. In all, ninety-three strains were tested; forty-five strains subcultured at daily intervals and forty-eight at longer or irregular intervals. Fifteen of the forty-five strains, which had been subcultured daily (Table 16, part 1), were found to be in Group A, having an LD 50 of less than 1×10^6. Of these, four were tested at the 2nd subculture and one at the 35th subculture, the remainder falling between these extremes. Similarly, twenty-two of the forty-eight strains, subcultured at irregular intervals, coming in Group A, were tested on the 2nd to the 15th subculture. Eighteen strains in the first series and ten in the second tested at the 2nd subculture were divided more or less equally between groups A, B, C and D. There is no evidence that the number of subcultures *per se* has any effect on the LD50. The assumption is made that strains in Group A have not degenerated.

(*e*) *Lapse of time between onset of disease and virulence*

There is no correlation (Table 17) between virulence for mice as measured by the LD50 and the time elapsed between onset of disease and testing in mice. Except for the special strains mentioned on p. 253, the time between onset and testing varied from 15 to 50 days with the forty-four strains tested. Strains with an LD50 of less than 1 million, which it is assumed will not have degenerated appreciably, ranged from the 20th to the 41st day.

Table 17. *Comparison of virulence* (*LD*50) *of* Haemophilus pertussis *strains and time elapsed from onset of disease to mouse test*

Figures in brackets 2nd and 3rd subculture only.

	Period between onset of disease and testing of strain in mice (days)																												
	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	41	43	50	
LD50 (millions)	No. of strains in each LD50 group																												Totals
<1	.	.	.	.	.	1	2	.	.	.	.	.	1	.	3	.	.	1	.	1	1	.	.	1	.	1	.	.	12
	.	.	.	.	.	(1)	(1)	.	.	.	.	.	(1)	.	(2)	.	.	.	.	(1)	(1)	.	.	.	.	.	.	.	(7)
1 and <5	1	.	.	.	.	1	.	.	.	3	.	.	.	1	1	.	.	.	.	.	.	.	.	.	2	1	.	.	10
	(1)	.	.	.	.	.	.	.	.	(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	(1)	(1)	.	.	(4)
5 and <25	.	.	1	.	.	.	.	.	1	.	.	.	.	1	.	1	.	.	2	1	1	.	1	.	1	.	1	1	12
	.	.	(1)	.	.	.	.	.	(1)	.	.	.	.	.	.	(1)	.	.	(2)	.	.	.	(1)	.	.	.	.	.	(6)
25 and <125	.	.	.	.	.	.	3	.	.	.	1	.	.	1	.	.	.	1	1	.	.	1	1	.	.	.	.	.	9
	.	.	.	.	.	.	(2)	.	.	(1)	.	.	.	.	.	.	.	.	.	.	.	(1)	.	.	.	.	.	.	(4)
>125	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	(0)
Totals	1	.	1	.	.	2	5	.	1	4	.	.	2	2	4	1	1	2	2	2	3	1	1	1	4	2	1	1	44
	(1)	.	(1)	.	.	(1)	(3)	.	(1)	(2)	.	.	(1)	(0)	(2)	(1)	(0)	(0)	(2)	(1)	(1)	(1)	(1)	(0)	(1)	(1)	(0)	(0)	(21)

(*f*) *Duration of disease at swabbing and virulence*

The same scatter was evident when the duration of the disease at the time of swabbing was compared with the virulence of the strain isolated. Strains of high virulence (Group A: LD50 less than $1{\cdot}0 \times 10^6$) were obtained from the 2nd day to the 21st day, and strains of low virulence (Group D: LD50, 25×10^6 to 125×10^6) from the 4th to the 21st day. There is no correlation between virulence and duration at least during the first three weeks of the disease.

(*g*) *Type of swab* (*prenasal or postnasal*) *and virulence*

It has been suggested that there is a difference between strains isolated from prenasal and postnasal swabs, particularly in colonial appearance (Evans, 1949). Though this may be so under certain conditions or on certain media, I have been unable to distinguish between the two and there was no difference in the virulence or the range of virulence of strains isolated by these two methods (Table 18).

DISCUSSION

Although *H. pertussis* is not a natural pathogen of mice and it is not possible to start an epidemic in mice even under laboratory conditions, mice can be infected artificially. Three main routes have been employed: (*a*) intraperitoneal

Table 18. *Comparison of virulence (LD 50) of* Haemophilus pertussis *strains and type of swabbing, postnasal or prenasal*

Strains isolated from					
Postnasal swabs			Prenasal swabs		
No. of strain of *H. pertussis*	No. of subculture	LD 50 (millions)	No. of strain of *H. pertussis*	No. of subculture	LD 50 (millions)
L9	2	0·5	L12	3	0·1
L11	3	0·75	L17	2	0·7
L10	3	1·0	L33	9	1·0
G928	4	3·1	L13	2	3·6
G942	3	16·5	L19	2	16·0
L3	2	86·0	L18	2	56·0
G947	6	143·0	L22	3	67·0

or intravenous; (*b*) intranasal; (*c*) intracerebral. Anderson & North (1943) showed that toxin is involved in the mortality in mice infected intraperitoneally, but it was left to Proom (1947) to clear up the relationship between infection by the intranasal and intraperitoneal routes. Proom showed that death following intraperitoneal injection of *H. pertussis* is due to a toxaemia which can be neutralized by specific antitoxin but not by a pure antibacterial serum, and that there is no evidence of the organism multiplying in the peritoneum or the blood stream. Infection by the intranasal route, first used by Burnet & Timmins (1937), results in an entirely different picture; something more nearly approaching an infection is obtained. The organisms multiply profusely in the lung and cause a pneumonia similar to that caused by influenza virus, etc. Good descriptions of the condition have been published (Hoyle & Orr, 1945; Lapin, 1943).

The intracerebral route, first used by Norton & Dingle (1935) and developed by Kendrick, Elderling, Dixon & Misner (1947), seems to be dependent on slightly different factors from the other routes, and a reference in Keogh & North (1948) to some unpublished observations of S. Fisher support this view. It is hoped to investigate these factors in greater detail in this laboratory.

The virulence of *H. pertussis* in mice is a variable and measurable character, and it is a pity that some workers have regarded it as a qualitative factor, a conception doubtless based on the view that Phase I organisms are virulent and Phase IV organisms avirulent (Leslie & Gardner, 1931). Virulence can be regarded as qualitative only if an arbitrary standard is set up.

The failure of some earlier workers (Toomey, Ranta & Takacs, 1935; Strean, 1943), using the intraperitoneal route in guinea-pigs or mice, to enhance the virulence of their strains to any great extent is explained by the work of Evans who reported that toxin production is one of the more stable characteristics of *H. pertussis*. Cruickshank & Freeman (1937) support this view; seventeen of their twenty-one freshly isolated strains had an average lethal dose of 6×10^9–9×10^9 organisms; their mice died usually on the 2nd–3rd days and rarely after the 5th day. Although it has been shown that death of mice after intraperitoneal injection of living organisms is principally due to the toxin contained in the injected organisms, other factors may play some part. It is otherwise difficult to understand the success of some workers (Cruickshank & Freeman, 1937; Elderling, 1942) in comparing the antigenic potency of different vaccines and the failure of rough vaccines to protect at least some mice.

The following conclusions can be drawn from the experiments reported here:

(1) Consistent and reproducible titrations of virulence can be made in mice by the intranasal route when precautions are taken to standardize procedure and to use mice of the same weight drawn from the same closely bred strain (Tables 1, 2, 3 and 6).

(2) Using mice of the strain C1 kept under the conditions specified, the LD 50 of a number of freshly isolated strains of *H. pertussis* varied from 0·1 to 152 million, calculated by the method of Reed & Muench on the 28th day. The wide variations in virulence of even the specially collected strains (Table 7) lead one to conclude that these variations in *H. pertussis* strains are true variations in the virulence of the strains, and not an artefact due to degeneration under laboratory conditions. This is not to deny that degeneration under laboratory conditions cannot or does not take place, for it obviously does, more quickly in some strains and more slowly in others. This degeneration or loss of virulence may depend to some extent on the medium employed and will probably vary in rate from one laboratory to another. The presence of this degeneration of pertussis strains is a complicating factor in virulence studies and means that while tests carried out with strains subcultured a few times permit a true evaluation of the virulence of the strain, those carried out on strains subcultured many times may or may not, and it is safe to consider them a true evaluation only in strains of high virulence where degeneration cannot have set in, that is strains in Group A. Strains of moderate virulence may be either strains of true moderate virulence or strains of high virulence which have degenerated to a moderate level; the same is true of strains of low virulence.

The passage of strains through mice and the testing of the virulence of the passaged strain may prove to be the best test for the true virulence of a strain. I found it impossible to enhance the virulence of a strain to any great extent by mouse passage except in the case of certain strains which are known to have lost virulence and in which virulence could be enhanced by mouse passage to a level approximately that of the original culture. Strains which showed the q.k.f. in early subculture but which had lost this factor, can regain it by mouse passage up to a point at which it is, apparently, lost permanently.

(3) Certain strains have the power of killing more rapidly than others (Table 9), though the meaning of this quick-killing factor is not yet understood. The difference between strains with and without this factor is statistically significant (Irwin, 1951, see Note). Preliminary experiments indicate that the q.k.f. is not antigenic. This factor can be lost on serial subculture in the laboratory and can in some cases be regained by mouse passage, but there is apparently a stage after which it is lost permanently; it does not appear possible to 'recover' this factor in strains which did not originally possess it.

(4) There was no correlation between virulence (LD 50) and the viable count (Table 11) or the toxin content of the suspension used to infect mice (Table 12).

(5) Nor was there any correlation between virulence and haemagglutinin (Tables 13 and 14). Keogh & North have suggested that the virulence of *H. pertussis* strains is related to their haemagglutinin content and that the strain with the highest haemagglutinin content shows maximal virulence; unfortunately they give details in their protocols of only seven strains. When, however, a number of Phase I organisms are examined it will be seen that virulence and haemagglutinin do not necessarily go hand in hand (Tables 1 and 14). While Keogh & North's findings are not disputed, it would be more correct to say that while the presence of haemagglutinin is a character of Phase I organisms, within this Phase it may or may not be present and it varies independently of virulence. With regard to toxin and haemagglutinin, it is, of course, possible that their action *in vivo* is not quite the same as their action *in vitro*. For example, toxin, which seems to be one of the more stable antigens of *H. pertussis*, might be released by certain strains which undergo autolysis more readily than others on introduction into the lung. This released toxin might act on the lung in some way and allow that strain to become established much more rapidly or successfully. This may equally be possible with haemagglutinin about whose action *in vivo* we have little information.

(6) There was no obvious correlation between virulence and the history of the strains tested, either in terms of the 'age' of the culture in number of subcultures, or the duration of illness at the time of swabbing, or the type of swab.

(7) There is no doubt that strains degenerate at different rates, and probably that all strains degenerate in time. It is reasonable to suggest that strains in group A—those with an LD 50 of less than $1{\cdot}0 \times 10^6$—have not degenerated or at any rate have not degenerated to any great extent.

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NOTE ON THE RELATION BETWEEN LD 50 AND AVERAGE TIME TO DEATH IN 25 STRAINS OF *H. PERTUSSIS*

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The values of the LD 50 at weekly intervals for 8 weeks are shown in Table 5 (p. 254). Clearly these values vary with the time at which they were calculated. A ratio of 3 to 1 is about significant, judging from Kendrick's tests (1948), and the Table shows greater variations than this at different times. Nevertheless, whenever the LD 50's are calculated they agree very well in the *order* in which they place the different strains. The coefficient of concordance, which would be unity for perfect agreement in order, is actually 0·85. Accordingly, the strains were classified into five groups of virulence as judged by the LD 50 at 56 days, as follows: less than 1 million; 1 million and less than 5 million; 5 million and less than 25 million; 25 million and less than 125 million; more than 125 million.

Two questions needed answering: (*a*) Does the relative rapidity of killing at different doses differ significantly in different strains? (*b*) If so, are these variations from strain to strain correlated significantly with virulence as judged by the LD 50? The answer to (*a*) was *Yes*, and to (*b*) *No*. The values of average time of death among the dead were examined for some of their differences and ratios. Suppose these averages for doses 100, 10, 1 and 0·1 millions are denoted by x_1, x_2, x_3 and x_4; the following values and their standard errors were calculated for each strain:

$$d_1 = x_2 - x_1, \qquad r_2 = d_2/l_2,$$
$$d_2 = x_4 - x_1, \qquad l_1 = \tfrac{1}{2}(x_2 + x_1),$$
$$r_1 = d_1/l_1, \qquad l_2 = \tfrac{1}{2}(x_4 + x_1).$$

The strains showed significant differences in d_1 and d_2 and in r_1 and r_2, but the average values of these quantities in the five virulence groups did *not* differ significantly. Further, there was no significant correlation between d_1 and d_2, or between r_1 and r_2. A high difference or ratio means that the strain kills *relatively* much more rapidly at the higher of the two doses compared. Table 1 shows the results in full.

Table 1

LD 50 (millions)	Strains of *H. pertussis*	d_2	d_2	r_1	r_2
<1	863	4·2	17·5	0·5915	1·2727
	L2	13·4	25·8	0·7090	1·0279
	1016	−2·5	16·4	−0·1832	0·7100
	1030	18·6	19·0	0·1273	1·1377
	1031	3·5	14·8	0·3955	1·0207
	1041	14·6	30·7	1·0355	1·3860
1 and <5	888	2·9	13·6	0·5421	1·2710
	928	5·3	21·1	0·8760	1·5125
	962	34·5	23·2	1·0764	0·8788
	983	4·0	10·0	0·1739	0·3846
5 and <25	894	4·0	7·0	0·6667	0·9333
	941	3·1	—	0·4960	—
	942	2·0	14·3	0·1575	0·7586
	978	15·2	−2·7	0·7876	−0·2609
	M6	21·4	17·8	1·0542	0·9622
	992	2·0	1·3	0·0952	0·0630
25 and <125	M1	24·9	7·9	1·1555	0·6054
	M2	0	—	0	—
	907	—	16·7	—	0·4963
	910	28·4	32·1	1·0216	1·0826
	M5	—	32·0	—	0·8421
	947	−29·6	9·4	−1·8734	0·2663
	959	26·7	11·2	1·2624	0·8485
	993	−6·6	—	−0·2292	—
	L3	8·8	−7·7	0·2543	−0·2922

Evidently the strains do show differences in relative rapidity of killing at different doses, but the precise nature of the differences is not very clear.

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The Isolation of Deoxyribonucleic Acid from Virulent and Avirulent Strains of *Haemophilus pertussis*

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SUMMARY: *Haemophilus pertussis* cells contain a relatively large amount of deoxyribonucleic acid which may be extracted from the organisms with 2 % sodium cholate.

A method is described for the isolation of deoxyribonucleic acid from the mechanically disintegrated organisms, which yields an apparently undegraded preparation free from ribonucleic acid and protein, but containing a polysaccharide component, probably in combination with the nucleic acid. This finding gives further evidence of the differences in biological properties of virulent and avirulent strains such as agglutinability by type-specific serum, precipitability by aluminium phosphate, and solubility in bile and sodium hydroxide.

Although the nucleic acid content of bacteria is relatively high compared with that of animal and higher plant tissues (Boivin, Tulasne & Vendrely, 1947; Belozerski, 1947), the isolation of bacterial nucleic acids, and particularly deoxyribonucleic acids, has been restricted by the difficulties of cultivation of the organisms in sufficient quantity and of disrupting the cells. In consequence, much of the earlier work on the deoxyribonucleic acids of bacteria (cf. reviews by Levene & Bass, 1931; Stacey, 1947) was concerned with the properties of small quantities of impure and considerably degraded material, often obtained by the use of strong alkalis. However, with the introduction in recent years of methods for the mechanical disintegration of micro-organisms, highly polymerized preparations of deoxyribonucleic acid have been isolated from yeast (Chargaff & Zamenhof, 1947, 1948) and an avian strain of *Mycobacterium tuberculosis* (Chargaff & Saidel, 1949) by mild procedures analogous to those developed initially by Mirsky & Pollister (1942) for the preparation of deoxyribonucleic acids from animal tissues.

The demonstration of the essential role of deoxyribonucleic acids in the transformation of pneumococcus types (Avery, McLeod & McCarty, 1944), and in the mutation of colon bacilli (Boivin, 1947), serves to emphasize the importance of a study of the properties of deoxyribonucleic acids isolated from micro-organisms under mild conditions. In initial experiments along these lines we attempted to isolate deoxyribonucleic acid from suspensions of *Micrococcus lysodeikticus* lysed with lysozyme, since Meyer & Hahnel (1946) observed that the high viscosity of such lysates was abolished by deoxyribonuclease. However, in our hands the yield of deoxyribonucleic acid from this source was negligible.

Recently Ungar & Muggleton (1949) showed that in addition to the well-known phase-specificity of *Haemophilus pertussis*, there are physico-chemical methods which differentiate between certain strains of *H. pertussis*. Virulent strains of *H. pertussis*, agglutinated only by specific phase I antiserum, are precipitated by suspensions of aluminium phosphate and are dissolved by 10 % sodium desoxycholate and sodium hydroxide solutions; in contrast, avirulent strains of *H. pertussis* are not affected by these reagents. Further, virulent strains form capsules in 24 hr. on Bordet-Gengou medium and require starch for their growth in fluid semi-defined medium (Ungar, James, Pegler, Muggleton & Tomich, 1950). It has also been observed that the material removed from *H. pertussis* by 2 % sodium cholate solution at 60° contains a high percentage of deoxyribonucleic acid (Webb, 1949). Together these observations seemed to indicate that the differences in the serological and physico-chemical behaviour of the two types of *H. pertussis* were based on differences in chemical composition. Using relatively large quantities of freeze-dried virulent and avirulent *H. pertussis* cultures, we have confirmed and extended these findings and have devised a method for the isolation of deoxyribonucleic acid from these sources in a comparatively undegraded form, free from all but traces of ribonucleic acid and protein.

In the present paper we describe the method of isolating the deoxyribonucleic acids. The main analytical data and physical properties of the preparations will be reported subsequently.

EXPERIMENTAL

Cultivation of the organisms

The virulent (83E) and avirulent (154E) strains of *H. pertussis* were grown in 1 l. screw-capped medicine bottles containing 250 ml. of semi-defined fluid medium consisting of salts, hydrolysed casein, soluble starch and yeast dialysate (Cohen & Wheeler, 1946). After 12 days at 37°, the organisms were collected by centrifugation, washed with saline and dried in the frozen state. The deoxyribonucleic acid content was *c.* 4 % (Table 1).

Although a prolonged period of growth was required to obtain the maximum

Table 1. *Phosphoprotein, deoxyribonucleic and ribonucleic acid content of the residual culture medium and of* Haemophilus pertussis *cells*

Determinations by the method of Schmidt & Thannhauser (1945). Results expressed as mg. P/50 mg. dry cells or evaporated culture medium.

	(mg. P/50 mg. dry material)		
Material	Deoxyribonucleic acid	Ribonucleic acid	Phosphoprotein
Virulent (83E) *H. pertussis* cells	0·214	0·412	Nil
Residual culture medium after growth of virulent (83E) cells	0·005	0·506	0·094
Avirulent (154E) *H. pertussis* cells	0·190	0·392	0·005
Residual culture medium after growth of avirulent (154E) cells	0·002	0·427	0·093

yield of organisms, no evidence of autolysis was observed in stained smears prepared from the cells at the end of the growth period, and chemical analysis of the residual culture medium after evaporation failed to show any significant amount of deoxyribonucleic acid (Table 1).

Extraction of Haemophilus pertussis *cells with 2 % sodium cholate solution at 60°*

The virulent (83E, 1·0 g.) and avirulent (154E, 0·85 g.) *H. pertussis* cells were extracted with 2 % (w/v) sodium cholate solution (100 ml.) at 60°, according to the method described by Henry & Stacey (1943, 1946). After 48 hr. the suspensions were centrifuged, the supernatants decanted (the extract of the avirulent organisms was extremely viscous and was obtained clear only after 4 hr. at 4500 r.p.m.) and the cell debris, in each case, washed with water and ethanol (twice) and dried with ethanol and ether. Absolute ethanol (4 vol.) was added to each of the aqueous sodium cholate extracts and the resulting precipitates (long fibres in the case of the extract from the avirulent organisms) were collected after 18 hr. by centrifugation, dehydrated with ethanol and dried with ethanol and ether. In this way 0·14 and 0·22 g. of material, which gave the usual qualitative tests for proteins and nucleic acids, were obtained from the extracts of the virulent and avirulent organisms respectively.

Table 2. *The distribution of ribonucleic and deoxyribonucleic acid in the nucleic acid fraction removed from* Haemophilus pertussis *cells by extraction with 2 % sodium cholate solution at 60°, and in the cell debris remaining after extraction*

Determinations by the method of Schmidt & Thannhauser (1945). Results expressed as mg. P/mg. dry nucleic acid fraction or cell debris.

	(mg. P/50 mg. sample)	
Material	**Deoxyribonucleic acid**	**Ribonucleic acid**
Nucleic acid fraction from sodium cholate extract of virulent *H. pertussis* (83E) (reducing value after acid hydrolysis = 27 %)	**0·470**	**0·254**
Virulent *H. pertussis* (83E) cell residue after sodium cholate extraction	**0·136**	**0·079**
Nucleic acid fraction from sodium cholate extract of avirulent *H. pertussis* (154E) (reducing value after acid hydrolysis = 7 %)	**0·600**	**0·298**
Avirulent *H. pertussis* (154E) cell residue after sodium cholate extraction	**0·035**	**0·097**

The analysis of these fractions by the Schmidt & Thannhauser (1945) procedure gave the results recorded in Table 2, which indicate that the extraction with sodium cholate removed most of the deoxyribonucleic acid of the cells. The material removed from the virulent organism (83E) under these conditions had a reducing value, after acid hydrolysis, equivalent to 27 % reducing sugar (as glucose), whereas the corresponding fraction from the avirulent organism yielded 7 % reducing sugar on hydrolysis (Table 2). Since this latter value must include the increase in reducing value due to the acid hydrolysis of the purine nucleotides, it is apparent that the extract from the virulent *H. pertussis* cells contained a polysaccharide component as well as protein and nucleic acids. It appears probable that the presence of this (specific) polysaccharide, mainly among the components of the sodium cholate extract of the virulent *H. pertussis* cells, is responsible, in some way, for the marked differences between the physical properties of the extracts from the virulent and avirulent organisms and for the differences in the ease of extraction of the cell components.

Isolation of deoxyribonucleic acid from Haemophilus pertussis

Although the procedure outlined above removed much of the deoxyribonucleic acid from the *H. pertussis* cells (Table 2), the alkalinity (pH 8) of the sodium cholate solution and the temperature at which the extraction was carried out probably caused some degradation of the nucleic acid molecule. In the following experiments, designed to overcome these difficulties, the dry bacteria were ground with 1·0 M sodium chloride solution to give a thick suspension, which was then shaken with the same volume of glass beads (ballotini) in a Mickle shaker (H. Mickle, Hampton, Middlesex) to disintegrate the organisms. Although the deoxyribonucleoprotein of *H. pertussis* was soluble in water and 0·14 M sodium chloride, the extraction was carried out with 1·0 M sodium chloride, since microscopic examination showed that more complete disintegration of the cells (60–70%) occurred under these conditions and resulted in higher yields of the nucleoprotein. Thus the yields of nucleoprotein obtained from 0·45 g. 83E cells, by the procedure described below, were respectively 75 and 35 mg. when the cells were disintegrated in 1·0 and 0·14 M sodium chloride.

Since it was considered that the polysaccharide previously encountered among the components of the sodium cholate extract of the virulent organism was located at the cell surface, attempts were made to remove this material, first by washing the intact organism with 0·14 M sodium chloride according to the method of Pittman (1931) for the removal of the soluble specific substance from *H. influenzae*. However, this procedure was abandoned, since the addition of ethanol to saline washings of virulent *H. pertussis* cells resulted in the immediate precipitation of long fibres which were easily removed from the granular precipitate, which separated more slowly. This fibrous precipitate gave a positive Dische (1930) reaction, and analysis by the Schmidt & Thannhauser (1945) method showed that it contained 6–7% deoxyribonucleic acid. Since previous observations indicated that little or no autolysis occurred during the cultivation of the organisms, the presence of deoxyribonucleic acid in the saline washings of *H. pertussis* cells may be of some significance in connexion with the location of this nucleic acid within the bacterial cell (cf. Boivin, 1948).

Preparation of crude nucleoprotein

One typical preparation will be described. The *H. pertussis* cells (10 g.) were dispersed in 1·0 M sodium chloride (200 ml.) by vigorous agitation in an 'Ato-Mix' homogenizer no. 800 (Measuring and Scientific Equipment Ltd., 14–28

Spencer Street, London S.W. 1). The resulting homogeneous suspension was allowed to stand at 0° overnight and then centrifuged. The supernatant (S_1) was decanted and stored at 0°. The residual cell suspension was diluted with 1·0 M sodium chloride (15 ml.), mixed with ballotini and shaken in the Mickle shaker. After 30 min. (3×10 min.) the suspension was diluted with S_1 (150 ml.), thoroughly mixed and centrifuged after 18 hr. at 0°. The supernatant (S_2) was decanted and the residual cells and debris were re-treated in the Mickle shaker as described above. This suspension was then diluted with S_1 (50 ml.) and centrifuged. The supernatant was combined with S_2 and absolute ethanol (1·5 vol.) added. The fibrous precipitate (D_1), which separated immediately, was rapidly removed from the accompanying granular precipitate (N) by winding around a glass rod. The precipitate (N), which gave a positive biuret and ninhydrin reaction, contained ribonucleic, but little deoxyribonucleic, acid (Table 3). The fibrous product (D_1) was transferred to 1·0 M sodium chloride (300 ml.) and stirred until no further material dissolved. The resulting suspension was centrifuged and the insoluble residue extracted for a further 24 hr. with 1·0 M sodium chloride (75 ml.) at 0°. After centrifuging, this extract was combined with the main solution containing the deoxyribonucleoprotein, and absolute ethanol (1·2 vol.) added. The resulting precipitate of long fibres (D_2) was removed from the granular precipitate as described above. This procedure of alternate solution in 1·0 M sodium chloride and precipitation with ethanol was repeated until the addition of ethanol yielded a fibrous precipitate only. By this method much of the accompanying ribonucleoprotein was removed from the fibrous material (Table 3). This crude deoxyribonucleoprotein, which gave positive biuret, xanthoproteic, Millon, Dische (1930) and Feulgen (1924) reactions, dissolved

Table 3. *Approximate nucleic acid content of fractions obtained during the isolation and purification of the deoxyribonucleic acids of* Haemophilus pertussis

Determinations by the method of Schmidt & Thannhauser (1945). Fractions dried with ethanol and ether before analysis.

	mg. P/50 mg. material			
	Virulent *H. pertussis* (83E)		Avirulent *H. pertussis* (154E)	
Material	Deoxyribonucleic acid	Ribonucleic acid	Deoxyribonucleic acid	Ribonucleic acid
Cell residue after extraction with 1·0 M sodium chloride	0·010–0·058	0·073–0·130	0·076	0·132
'Granular' fraction (N)	0·048	0·415	0·030	0·500
Nucleoprotein (D_1)	0·570	0·481	0·485	0·471
Nucleoprotein (D_3)	0·632	0·271	—	—
Nucleoprotein (D_5)	0·910	0·195	0·820	0·210
Protein moiety removed from nucleoprotein by Sevag's (1934) method	0·089	0·105	0·105	0·143

rapidly in water, or in 1·0 M sodium chloride, to give a viscous solution. It was not, however, precipitated from its solution in 1·0 M sodium chloride on dilution with water and, as recorded above, was soluble in 0·14 M sodium chloride. In this respect the deoxyribonucleoprotein of *H. pertussis* differs from the deoxyribonucleoproteins of animal tissues (Mirsky & Pollister, 1942) and from the nucleoprotein isolated by Mirsky & Pollister (1946) from Type III pneumococci, but resembles the deoxyribonucleoprotein of avian tubercle bacilli (Chargaff & Saidel, 1949).

Dissociation of the nucleoprotein: isolation of deoxyribonucleic acid

Prolonged dialysis of a solution of the nucleoprotein in 1·0 M sodium chloride against 1·0 M sodium chloride at 2° (cf. Pollister & Mirsky, 1946), or saturation with sodium chloride (cf. Hammarsten, 1924; Chargaff & Saidel, 1949) failed to dissociate the nucleoprotein. Accordingly, the protein moiety was removed from the nucleoprotein by the Sevag (1934) method, a procedure which resulted in some loss of deoxyribonucleic acid (Table 3). After eight treatments with chloroform octanol mixture (9:1) the saline solution was free from protein. Addition of ethanol (4 vol.) resulted in the separation of fibres together with a granular precipitate. The fibrous material was collected and purified by repeated solution in 1·0 M sodium chloride followed by precipitation with ethanol until the final precipitation yielded fibres only. This precipitate was dissolved in distilled water and the solution dialysed for 24 hr. at 2° against frequent changes of distilled water. Finally, the nucleic acid was recovered as white fibres by ethanol precipitation or by evaporation in the frozen state *in vacuo*.

The nucleic acids thus obtained from both the virulent and avirulent organisms were free from detectable amounts of protein. Furthermore, the analysis of the preparations by the method of Euler & Hahn (1946) failed to reveal the presence of ribonucleic acid. Owing to the small quantities of nucleic acids available (Table 4), these analyses were restricted to 10 mg. quantities. Thus, the presence of traces of protein and ribonucleic acid (<2 %) in the preparations cannot be excluded. However, the low content of ribonucleic acid

Table 4. *Some properties of the deoxyribonucleic acids of virulent and avirulent strains of* Haemophilus pertussis

	Deoxyribonucleic acid from	
	Virulent *H. pertussis* (83E)	Avirulent *H. pertussis* (154E)
Yield (mg./10 g. cells)	125	200
Absorption maximum (mμ.)	258	258
Dische (1930) coloration extinction/mg. P	3·78	3·83
Nitrogen (%)	10·83	10·23
Phosphorus (%)	6·36	5·65
N/P ratio	1·7	1·8
Reducing value % (as glucose) after acid hydrolysis	31	33

in the deoxyribonucleic acid preparations, as suggested by these analyses, is of considerable interest when considered in relation to the simple fractionation technique described in this paper, and the difficulties which often attend the separation of mixtures of the nucleic acids (cf. Laland, Overend & Webb, 1950).

The deoxyribonucleic acid preparations from both the virulent and avirulent organisms contained a polysaccharide component, as indicated by the low analytical figures for nitrogen and phosphorus and by the high reducing values shown after acid hydrolysis (Table 4). The polysaccharide(s) appeared to be firmly bound to the nucleic acids, since the addition of acetic acid or 1 % (w/v) lanthanum acetate to aqueous solutions of the above preparations resulted in the complete precipitation of both polysaccharide and nucleic acid.

Methods for the separation of the carbohydrate component and the immunological examination of the various fractions isolated in the present work are under investigation.

DISCUSSION

The differences in chemical composition between the virulent and avirulent cells examined will readily be seen from Tables 1 to 4. Further work is needed to emphasize the significance of these differences, but one particular observation may be of some importance, namely, the fact that the deoxyribonucleic acid is more difficult to extract from the virulent cells. One explanation of this is that it is firmly attached to a polysaccharide which is present in higher proportions possibly at the surface of the virulent cells.

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Shaking Apparatus for the Aeration of Bacterial Cultures

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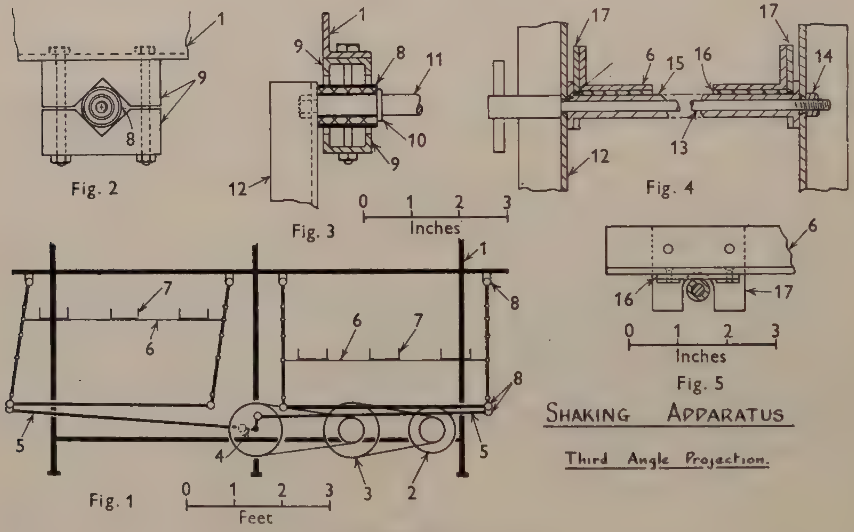
A large shaker designed to satisfy simultaneously and without mutual interference the conflicting demands of a team of bacteriologists, has been placed in one of this Institute's hot-rooms. The shaker runs at a constant speed of 100 shakes/min., but each user is free to select an amplitude of reciprocative motion to suit his requirements, by placing a loaded tray at a suitable distance from the centre of oscillation. This can be done without stopping the motion of the apparatus.



Figs. 1–5

It consists of an aluminium alloy scaffolding (1) (Fig. 1, thick lines) and on to this are hinged two 'swings' (medium lines) which are driven at constant speed by means of a 1500 r.p.m. synchronous motor (2). This, through a 2-stage 'Vee' belt reduction gear (3), drives a two-throw crank (4) at 100 r.p.m. The crank pins are 90° out of phase with each other and oscillate one swing each by means of long connecting rods (5). Loose bearer rails (6) can be attached to the swings at various distances from the upper hinge points and reciprocate therefore at various amplitudes (maximum ± 2 in.). Small trays (7), loaded in the laboratories, can be placed securely on the bearer rails without stopping the machine.

Design details are illustrated by subsequent figures. Fig. 2 shows a typical hinge as used on the swings, and the swing end of the connecting rods. It consists essentially of a 'Silentblock', i.e. a rubber bush pressed in between two steel sleeves, the outer one being pinched by two 'Vee' notches in short pieces of aluminium alloy channel (9) and the inner one is screwed against a shoulder (10) on a bar (11). As may be seen more clearly in Fig. 3, the channels (9) are bolted to the scaffolding (1) and the bar carries the vertical members (12) of the swings.

Fig. 4 describes the method used in locating the bearer rails. Pins (13) are inserted into holes in the front vertical members (12) and screwed into 'Hank Bushes' (14) secured to the other vertical members.

Holes for pins (13) are provided at exponentially increasing distances from the upper hinge. The distance increases from hole to hole by 50 % and, of course, the amplitudes of reciprocation are increasing correspondingly.

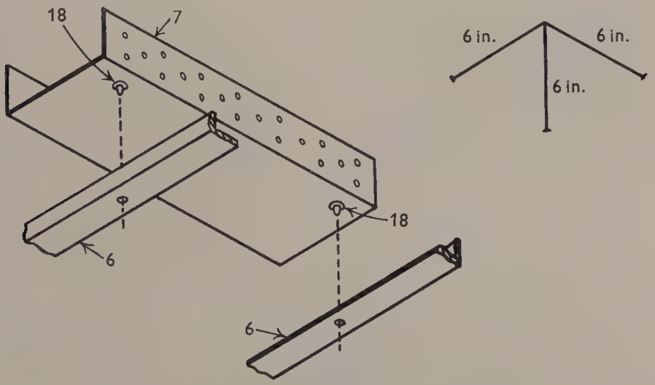


Fig. 6

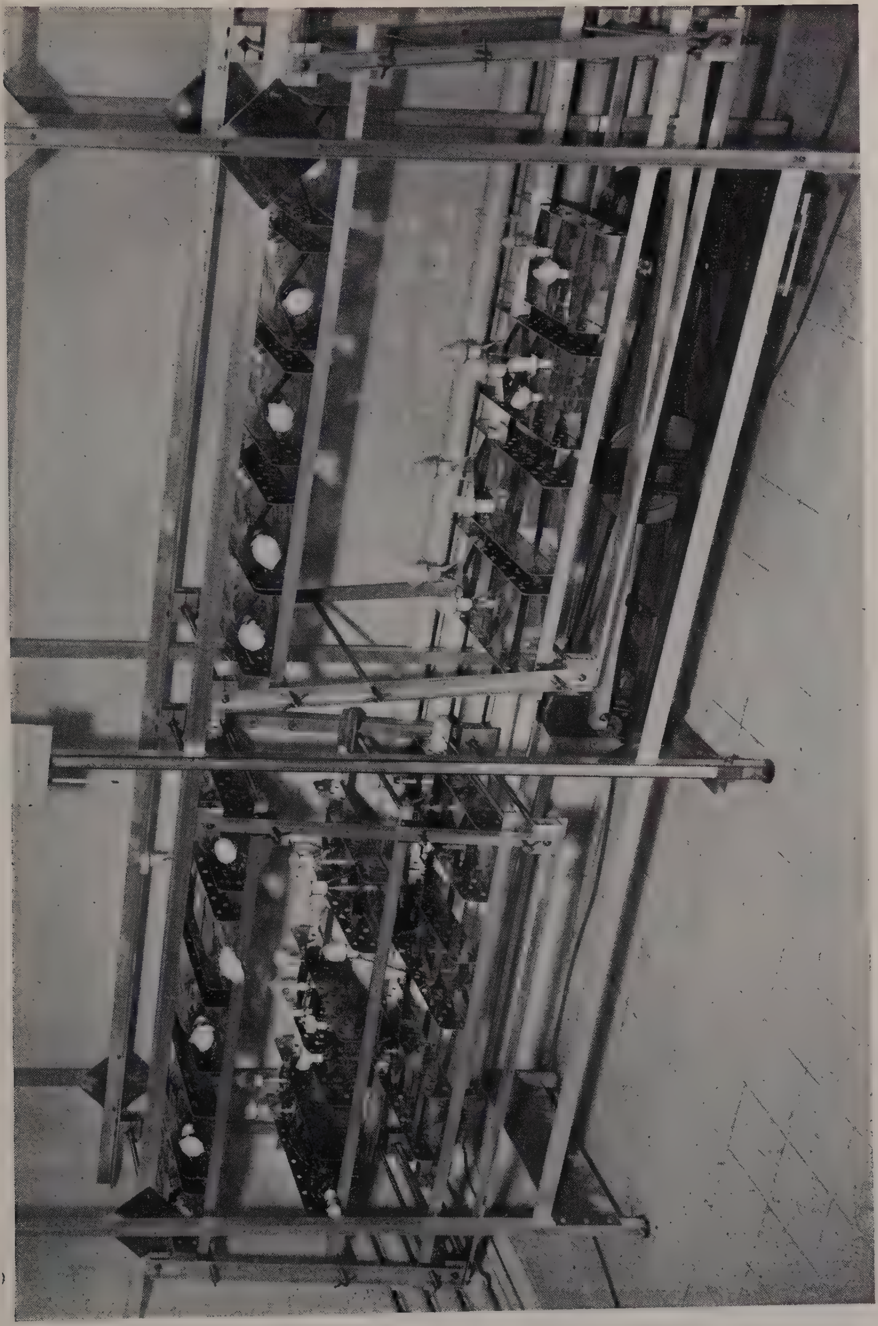
Tubular spacers (15) are threaded on the pins (13), and the bearers (6) rest on these. (It was originally intended that 15 should rotate on 13. This did not take place however. In future 15 will be omitted and 13 made correspondingly thicker.) A piece of rubber sheet (16) is secured to the underside of the bearer rail (6) and at one end check plates (17) are provided to prevent the bearer rail wandering off their supports. Rubber treads and check plates are seen better in Fig. 5.

Fig. 6 shows in isometric projection a typical tray (7) formed of (pure) aluminium sheet. This is equipped with two conical feet (18) by means of which it engages holes spaced 8 in. apart in the bearer rails (6). A great number of holes ($\frac{3}{8}$ in. diameter) are provided in the sides of the trays. Rubber bands threaded through and secured by office paper-clips (Gem Clips) are used to secure bottles and flasks to the trays.

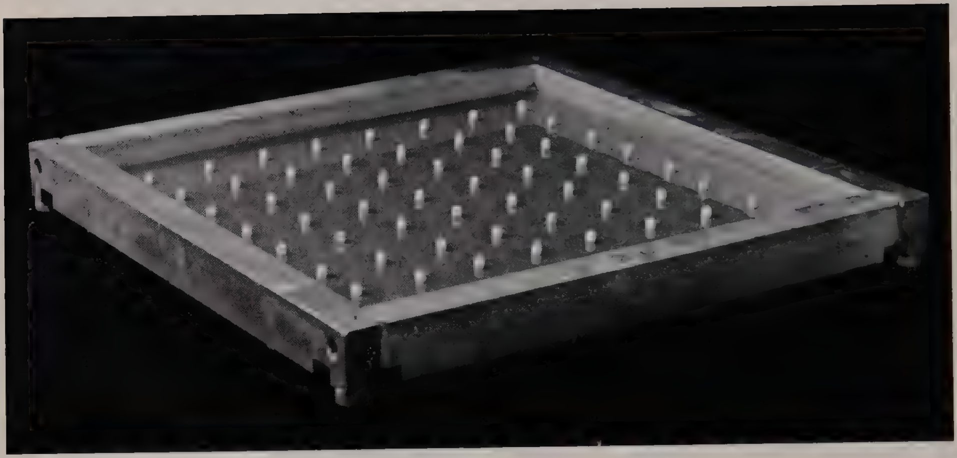
It was found that although a $\frac{1}{8}$ h.p. motor provided adequate torque, it became too hot at the prevailing temperature (37° ambient) and a $\frac{1}{4}$ h.p. motor was substituted. The photograph (Pl. 1), shows the part of the apparatus represented diagrammatically by Fig. 1. In case of need a second pair of swings can be added above the part shown in a space occupied at present by (stationary) shelves. The apparatus is so free of noises that provided there is no jingle of caps the prevailing sound is the swish of the bottle contents.

The development owes much of its success to the skill and ingenuity of Mr Beadle and Mr Davey, respectively, the instrument-maker and the foreman in charge of the project.

(*Received 7 June* 1950)



O. KANTOROWITZ—SHAKING APPARATUS FOR BACTERIAL CULTURES. PLATE 1



O. KANTOROWITZ (pp. 357–359)—ANTIBIOTIC ASSAY TRAY. PLATE 1

FLEWETT, T. H. & CHALLICE, C. E. (1951). *J. gen. Microbiol.* 5, 279–286.

The Intracellular Growth of Fowl-Plague Virus

A Phase-Contrast and Electron Microscopical Study of Infected Tissue Cultures

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SUMMARY: The changes in fowl-plague infected tissue cultures have been investigated by the phase-contrast and electron microscopes. The nucleolus of infected cells first increased in size and then became granular and highly refractile, finally disintegrating into fragments about 64 hr. after infection. After 36–48 hr., when the inclusions were already developed, filaments were found, usually in bundles, possibly having escaped from the nucleus into the cytoplasm; a few filaments showed segmentation into spherical bodies and shorter filaments. A possible life cycle is suggested for the virus: first, penetration of the nucleus and growth within it, then extension into the cytoplasm in a filamentous form, and eventual segmentation of some of the filaments into elementary bodies.

It was shown by Porter, Claude & Fullam (1945) that satisfactory electron micrographs could be obtained of the cytoplasm of well-extended chick fibroblasts. Subsequently it was demonstrated that intracellular virus particles, as small as 60mμ. in size, could readily be resolved (Claude, Porter & Pickels, 1947; Bang & Gey, 1948, 1949; Porter & Thompson, 1947, 1948). The use of the phase-contrast microscope in the study of viral inclusion bodies was demonstrated by Angulo, Richards & Roque (1949) and Angulo (1949) for a variety of viral infections.

When Chu, Dawson & Elford (1949) demonstrated such long and numerous filamentous forms in recently isolated strains of A-prime influenza, it seemed that the direct examination of infected tissue-culture cells might give some information as to whether these filamentous forms represented a stage in the life cycle of the virus.

The morphology of fowl-plague virus is very similar to that of influenza A in that filaments and elementary bodies of the same size (Dawson & Elford, 1949) occur in infected allantoic fluid. The virus is highly pathogenic for chick embryo tissues; infection can easily be recognized by the presence of intranuclear inclusion bodies; and the virus is pantropic for chick cells of all kinds (Findlay, Mackenzie & Stern, 1937). It is therefore suitable for an investigation of this type.

MATERIALS AND METHODS

One of the strains of fowl-plague virus used was obtained from Dr F. Doyle, and is the strain used by Dawson & Elford (1949); the other we isolated from the dried liver tissue of a fowl from an epizootic in France, kindly sent by Dr Lépine. Nearly all the observations were made with the first strain; the second took slightly longer to kill chick embryos, but in all other respects appeared to behave similarly. The tissues were pieces of amnion and pectoral muscle from 10-day-old eggs and the nutrient medium was amniotic fluid from normal 10-day eggs.

The fluid used to infect the tissue culture was prepared by inoculating 10-day eggs by the amniotic route with a 1/1000 dilution of allantoic fluid containing fowl-plague virus with a haemagglutination titre of 2500 to 10,000. The eggs were incubated for 18 hr. at 37°. After chilling overnight at 4°, the amniotic fluid was harvested; 1 part of Hartley's broth containing 10 % normal horse serum was added to 4 parts of the infected amniotic fluid, and the mixture was then filtered through a 'Gradocol' membrane of average pore diameter 0·65–0·7 μ., in order to remove the greater part of the filamentous forms of the virus. Preparations of virus before and after filtration were made on haemolysed chick erythrocytes by the method of Dawson & Elford (1949) and photographed in the electron microscope; they showed that only short filaments up to 1 μ. in length and spherical particles were present in the filtrate. The haemagglutination titres of the filtrates were determined, using 0·5 % of chick erythrocytes, with a final volume of 0·5 ml. in each dilution. The sedimentation patterns were read after 1 hr. at room temperature, and the titres varied between 1/20 and 1/80 in different preparations. The filtrates were stored in 0·5 ml. amounts at −76° until required.

At first we tried the methods of Porter *et al.* (1945) for preparing cultures for the electron microscope; but great difficulty was encountered in detaching the 'Formvar' membrane from the cover-slip. The following method was found to give consistent and satisfactory results:

A Petri dish was filled with Simms's solution, adjusted to a pH about 7·4. A drop of a 1 % solution of 'Formvar' (polyvinyl formal) in ethylene dichloride was allowed to fall on to its surface; the drop spread and the solvent evaporated to leave a thin membrane. The solution of 'Formvar' appears to be self-sterilizing. This membrane was then picked up on a cover-slip so that it overlapped the cover-slip all round. Three or four small fragments of tissue (about ½ mm. square) were placed on the membrane in a small drop of amniotic fluid. The cover-slip was inverted over a hollowed slide, sealed with paraffin and incubated for 2 days as a 'lying-drop' (Fig. 1). It was then examined to be sure that outgrowth had occurred. The cover-slip, with the attached membrane, was removed with a hot scalpel, and a drop of infected amniotic fluid was added to the culture. The cover-slip was then sealed to the hollowed slide again, and the preparation returned to the incubator. The cultures were examined at intervals by the phase-contrast microscope, and specially thin areas were selected for mounting for the electron microscope.

For mounting, the culture was opened as before, and Simms's solution was run under the edge of the membrane until it was raised from the surface of the cover-slip. The culture was washed carefully with Simms's solution to remove as much free protein as possible, and then a drop of a 2 % (w/v) solution of osmium tetroxide in water was added, and left to fix for 2 hr. The fixation was complete quite quickly, but the prolonged action of the osmium tetroxide increased contrast slightly.

A horseshoe-shaped piece of glass rod 5 mm. in diameter was placed on the membrane to hold it in position, so that the culture lay between the ends of the horseshoe. The culture was washed in several changes of double-distilled water, and the membrane was floated upwards so that it was about 2 mm. above the cover-slip (Fig. 2). An electron microscope grid of 200-mesh was pushed into position between the membrane and the cover-slip with a glass rod so that the cells to be examined lay over the central squares of the grid. The distilled water was then aspirated from beneath the grid so that the membrane gradually sank on to it. The position of the cells with respect to the grid was controlled under the microscope during the mounting; only the central nine squares of the grid were available for examination. The preparation was allowed to dry and was examined in the microscope. The techniques of mounting tissue cultures for the electron microscope will be more fully described elsewhere.

Most of the electron micrographs were taken with the Siemens microscope at a magnification of about 5000, at 75 kV. and using a 70 μ. objective aperture. It was found, however, that better definition of thicker parts of the cell near the nucleus was obtained at 100 kV. using a 20 μ. objective aperture. Some photographs were taken with the R.C.A. type E.M.B. microscope at 60 kV. with no objective aperture, at a magnification of 11,000.

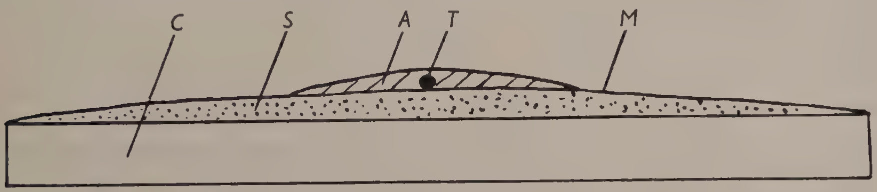

Fig. 1. *A*, amniotic fluid; *C*, coverslip; *M*, membrane; *S*, Simms's solution; *T*, tissue explant.

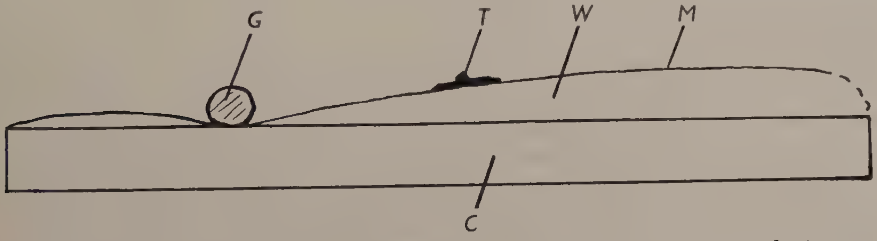

Fig. 2. *C*, cover-slip; *G*, glass horseshoe; *M*, membrane; *T*, tissue explant; *W*, distilled water.

RESULTS

When amnion was cultured, the growth was mainly epithelial; fibroblasts were supplied from fragments of lung or pectoral muscle. Some growth continued after the addition of virus, and mitoses could be seen even 48 hr. after inoculation.

With the phase-contrast microscope the first change visible after infection was an increase in the size of some of the nucleoli, both in epithelium and in fibroblasts (Pl. 1, fig. 2; compare with normal in fig. 1). This change was first visible in fibroblasts about 24 hr. after infection (Pl. 1, fig. 3), and in epithelium 36 hr. after infection (Pl. 1, fig. 4). After 48 hr. the change was more marked, and in addition, many of the affected nucleoli became much more refractile (Pl. 1, fig. 5) and instead of the smooth homogeneous appearance of the normal nucleolus, they acquired a distinctive structure almost micro-crystalline in appearance. In many nuclei, highly refractile fragments, presumably derived from the altered nucleolus, were seen in the nucleoplasm (Pl. 1, fig. 6). Some of the altered nucleoli grew to occupy more than half of the nucleus; they probably constitute the intranuclear inclusions described by Findlay *et al.* (1937) and other authors. The nuclear membrane then became invisible. Occasionally the whole inclusion broke up (Pl. 1, fig. 7).

After 48 hr. incubation, the outgrowth already looked 'moth-eaten', where cells had fallen out, and amorphous cellular debris could be seen floating in the culture medium. About 64 hr. after inoculation, many more of the cells showed intranuclear inclusions and the disintegration of the outgrowth was much more advanced; after 72 hr. little was left of the outgrowth and the supernatant fluid was thick with debris.

Although nuclear changes might be advanced, the mitochondria showed little change. They appeared, 24 hr. after inoculation, perhaps larger than normal; after 48 hr., some were somewhat shorter and thicker, with clubbed ends. Only when the cell was about to disintegrate did the mitochondria become rounded up.

Small granular inclusions were sometimes seen in the cytoplasm near the nucleus; but they occurred in only a few of the cells; and as similar structures were very occasionally seen in uninfected preparations, their nature is uncertain.

As the infection progressed, many of the infected cells, especially epithelium, became vacuolated. The vacuoles were concentrated around the nucleus; small particles in rapid Brownian motion could be seen within them. These vacuoles were not as refractile as the fat occurring in cells grown in a medium of high protein content; a few small highly refractile fat droplets were usually seen, but did not increase in number with infection.

Cultures were mounted for the electron microscope 6, 12, 18, 24, 36, 48, 64 and 72 hr. after inoculation with virus. No virus particles could be seen at all until after 36 hr., when filaments of virus were found in the cytoplasm of fibroblasts, occurring in bundles of a few filaments, up to 2μ. in length. These filaments, in some cases at any rate, appear to come from the nucleus; Pl. 2, fig. 8, shows a bunch of filaments apparently emerging from the nucleus of an epithelial cell. No elementary bodies could be seen at this stage.

After 48 hr., in most preparations, long bundles of filaments up to 7μ. in length, with many separate individual filaments, each about $0{\cdot}1\mu$. thick, could be found (Pl. 2, figs. 9, 10; Pl. 3, figs. 11, 12). Some of them could be seen apparently undergoing segmentation into spherical bodies and into shorter filaments. The bundles of filaments were mostly situated near the nucleus, but a few were found in the processes of fibroblasts. No branching filaments were discovered. There were no colonies of spherical elementary bodies and only a few isolated elementary bodies were found, and then only in association with the long forms.

In the cytoplasm of fibroblasts peculiar spherical structures, about 3 μ. in diameter, with areas of greater density within them, were occasionally found (Pl. 3, fig. 12). No definite virus particles could be made out within these structures, and they were never found in control material. Their significance is unknown. Virus filaments bent in an acute angle showed a swelling at the 'elbow' (Pl. 2, fig. 10).

Finally, as the cell disintegrated, the regular outline of the cytoplasm was lost, and amorphous debris was seen with large numbers of filaments lying in and around it; many of these showed segmentation into shorter elements and occasionally into spherical bodies of the same thickness; and a few isolated spherical elementary bodies were found (Pl. 3, fig. 13). One fibroblast, 64 hr. after inoculation, showed an area of greater density at the edge of the cytoplasm with many long filaments radiating from it, suggesting in appearance a colony of virus particles in the act of rupture (Pl. 3, fig. 11).

The nucleus was always so thick as to make it almost opaque to electrons. The nucleolus could usually be made out, but the detail which was resolved was no more, and indeed usually less than could be seen by the phase-contrast microscope.

When fixation with osmic acid was limited to 30 sec., with a 20 μ. objective aperture and an accelerated potential of 100 kV. much more detail could sometimes be made out in the nucleus; but the electron scattering was so great that virus particles could not have been made out in it.

By hydrolysis for 7 min. at 56° in N-HCl, the area around the nucleus could be made more transparent to electrons; Pl. 2, fig. 10 and Pl. 3, fig. 12, are of preparations so treated. Fowl-plague and influenza viruses adsorbed upon chick red cells showed no apparent change after this treatment when examined in the electron microscope.

We have always found the cytoplasm in both epithelium and fibroblasts to show only finely reticular structure; the coarse fibrils described by Bang & Gey (1948) in rat fibroblasts were not seen. Excessive washing with Simms's solution may, however, induce the appearance of coarse reticulation; and no washing at all may result in the precipitation of threads, presumably fibrin, which may simulate virus filaments. These fibrin threads may be recognized by their tapered ends, their irregular thickness—usually 50 mμ. or less—and their relatively low contrast compared with virus particles.

Many attempts were made to demonstrate influenza virus within infected cells by the method described above. A few electron micrographs were obtained showing filamentous structures within cells, Pl. 3, fig. 14, but we have not been able to repeat these results.

DISCUSSION

The question arises as to whether the filaments and elementary bodies occurring within infected cells are, in fact, fowl-plague virus particles. The evidence that they are is:

(1) The particles and filaments are the same diameter as fowl-plague virus.

(2) They are only found in infected cells and never in control preparations.

(3) Their appearance is associated with the presence of inclusion bodies in the cultured cells and the subsequent destruction of the outgrowth.

(4) Multiplication of virus in the tissue cultures can be demonstrated by other means.

Filamentous structures which might simulate virus are collagen and fibrin strands. Collagen, like the filaments described here, occurs in bundles (Heinmets & Golub, 1948) and some of the collagen fibrils they illustrated are of about the same thickness as ours. But we have never seen the characteristic regular spacing of the transverse bands of collagen at 65 mμ. as described by them; and furthermore, long filaments appear in infected epithelial cells growing in sheets in which there were no fibroblasts; epithelium does not produce collagen. Mitochondria are readily distinguished by their much greater size and less distinct outline.

The uniform thickness, the segmentation, the peculiar angulation, the relatively high electron-density and the strictly intracellular position in the earlier stages of growth all suggest that they are not fibrin. The similar filaments occurring in infected allantoic fluid resist digestion with crude pancreatic extract containing trypsin and lipase.

It seems likely then, that these structures do in fact represent the virus itself; and if so, they must have developed within the cell from the elementary bodies and the very short rods present in the original inoculum. What happens in the early stages of intracellular growth of virus is unknown; no virus is found in the cytoplasm until the intranuclear inclusions are well developed; and when it is found, it is in the form of long filaments already arranged in bundles, and there is no direct evidence of the growth of these from the elementary bodies of the inoculum. A tentative hypothesis might be put forward that fowl-plague virus penetrating a cell first invades the nucleus, multiplying in or in the region of the nucleoli, and that only after a certain stage has been reached does it extend into the cytoplasm in a filamentous form.

The segmentation of the filaments is associated with the disintegration of the infected cell, and it seems possible that segmentation might be due to the action of enzymes released at this stage. Mixed enzymes from bovine pancreas have no demonstrable effect, and unless there are special enzyme systems in the infected cell induced or modified by the infection, it must be assumed that the segmentation is spontaneous.

The association of filamentous forms with the elementary bodies has recently been described independently in the case of bacteriophage by Herčik (1950) and Wyckoff (1950); it was there suggested that the filaments might be aberrant forms of virus developing in an unsuitable environment. That is not likely to be the case with fowl-plague developing in the embryonic tissue of the natural host; it would, however, be interesting to examine the pericardial fluid from an adult bird dying of fowl-plague. If one elementary body should grow into one filament, no increase in infectivity would be expected, while there might be a considerable increase in amount of antigen as determined by complement fixation until segmentation into elementary bodies took place.

ADDENDUM

Murphy, Karzon & Bang (1950) have just published a paper illustrating filamentous forms of PR8 influenza virus in disintegrating cells of infected tissue cultures. They consider, as we do, that the filamentous forms probably undergo segmentation into elementary bodies.

We would like to thank Dr I. M. Dawson, with whose collaboration one of us (T. H. F.) devised the technique described; Drs C. H. Andrewes and W. J. Elford for their advice and encouragement; Mr J. Smiles for some of the phase-contrast photographs; and Dr Lépine for a strain of fowl-plague virus.

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DESCRIPTION OF PLATES

Plate 1. All × 1500

Fig. 1. Nucleus and mitochondria of a normal epithelial cell. The nucleoli are small and not very refractile. The nuclear membrane is clearly defined. The mitochondria are shorter than usual.

Fig. 2. Nucleus and filamentous mitochondria of epithelial cell 24 hr. after inoculation with fowl-plague virus. The nucleus is greatly enlarged.

Fig. 3. Nucleus of fibroblast 24 hr. after inoculation. The nucleolus is enlarged and more refractile than normal; the mitochondria have become rounded up.

Fig. 4. Nucleus of epithelial cell 36 hr. after inoculation. The nucleolus is highly refractile› as indicated by the halo, although not much larger than normal.

Fig. 5. Swollen granular intranuclear inclusions; epithelium 48 hr. after inoculation. The mitochondria have almost disappeared.

Fig. 6. Nuclei of epithelial cells 48 hr. after inoculation. The inclusions are beginning to break up into the small granules into the nuclei. The cytoplasm is vacuolated and the mitochondria poorly defined.

Fig. 7. Fibroblast, 48 hr. after inoculation. The inclusion has disintegrated into fragments. The mitochondria have disappeared. The arrow indicates the nucleus.

Plate 2

Fig. 8. Filaments escaping from nucleus of an epithelial cell 48 hr. after inoculation. (The nucleus is the dense area at the top.) The filaments appear broader in the vacuole than in the thickness of the cytoplasm. The arrow indicates a mitochondrion. 100 kV., 20 μ. objective aperture. × 10,000.

Fig. 9. Bundles of filaments in the cytoplasm of an epithelial cell, 48 hr. after inoculation. One filament is segmented into spherical particles. The cytoplasm at the top has been torn in drying. 75 kV., 70 μ. objective aperture. × 10,000.

Fig. 10. Filaments in fibroblast, 48 hr. after inoculation. One filament shows 'elbow' swelling. 70 kV. × 22,000.

Plate 3

Fig. 11. A group of filaments escaping from an infected fibroblast, 64 hr. after inoculation. 75 kV., 70 μ. objective aperture. × 10,000.

Fig. 12. A peculiar spherical structure occasionally seen in infected fibroblasts. The filaments in the picture show segmentation. × 22,000.

Fig. 13. Cytoplasmic debris, filaments and a few elementary bodies. Disintegrating fibroblast, 72 hr. after inoculation. × 10,000.

Fig. 14. Cytoplasm of epithelial cell, 24 hr. after inoculation with Nederland 1 strain of influenza A-prime virus. × 10,000. (Electron micrograph by Dr I. M. Dawson.)

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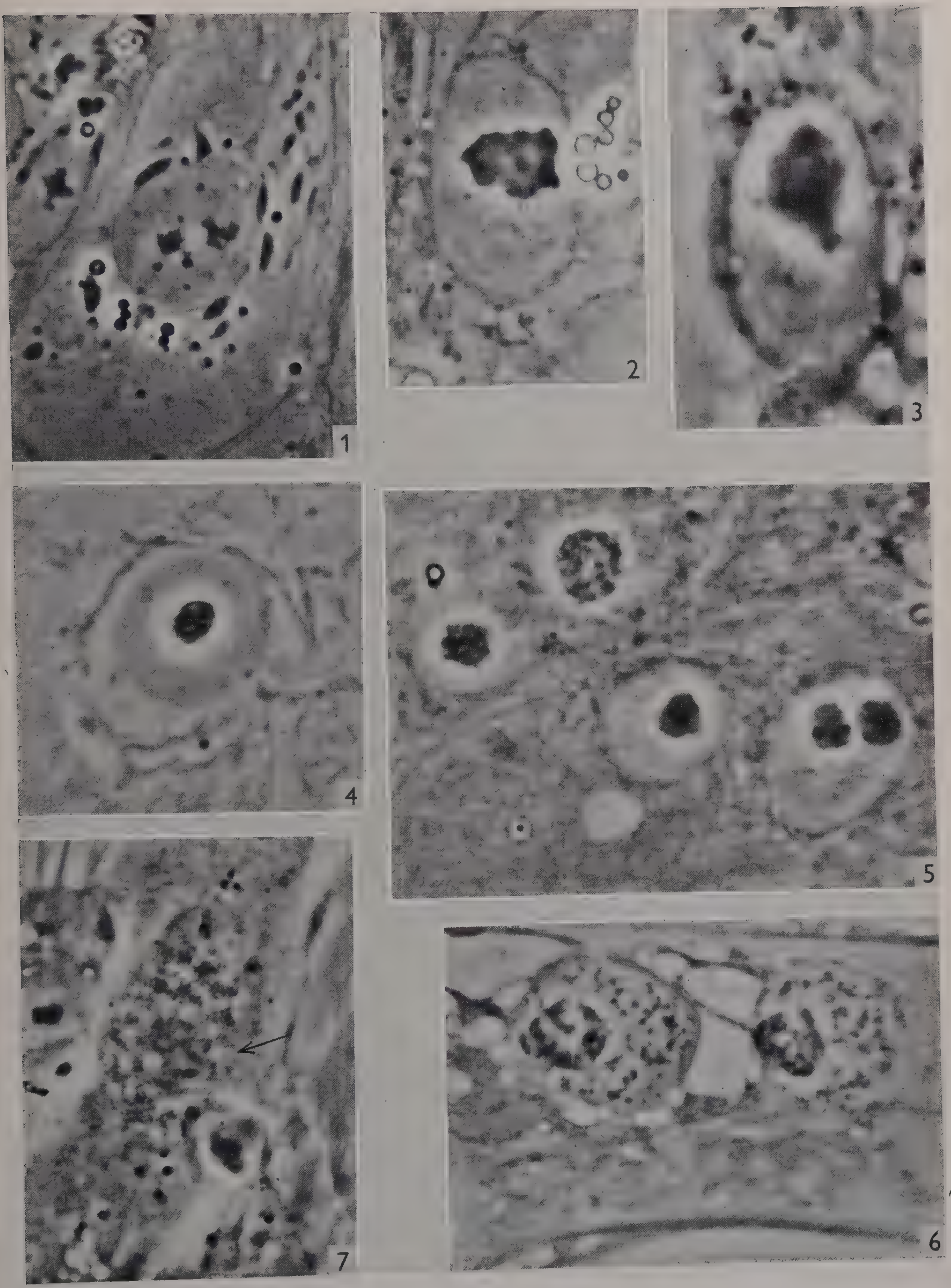
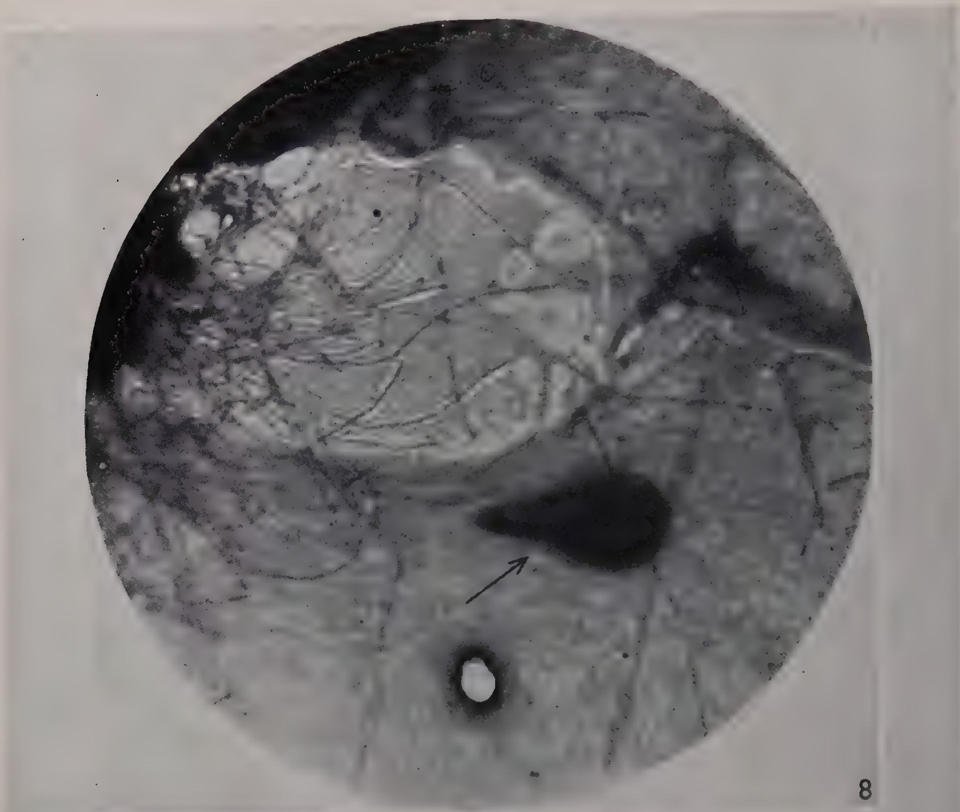


T. H. Flewett & C. E. Challis—Intracellular growth of fowl-plague virus.
Plate 1



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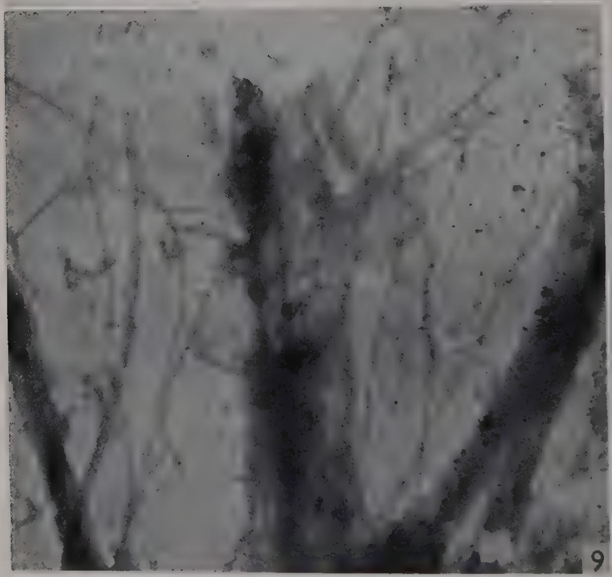

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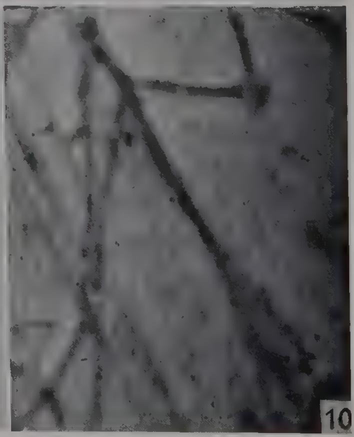

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T. H. Flewett & C. E. Challis—Intracellular growth of fowl-plague virus.
Plate 2

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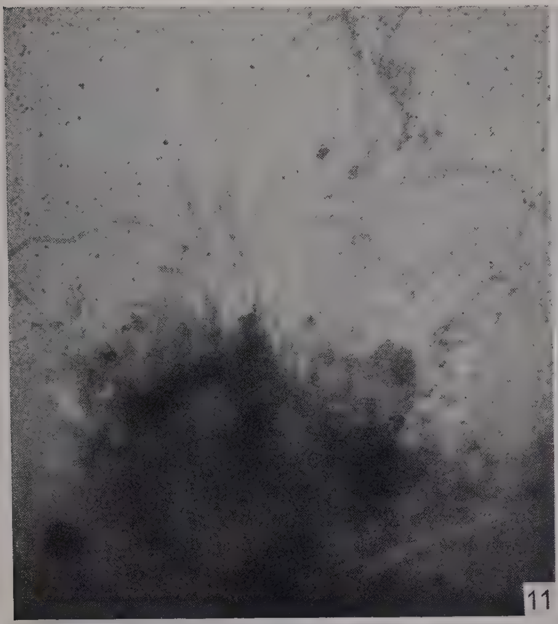
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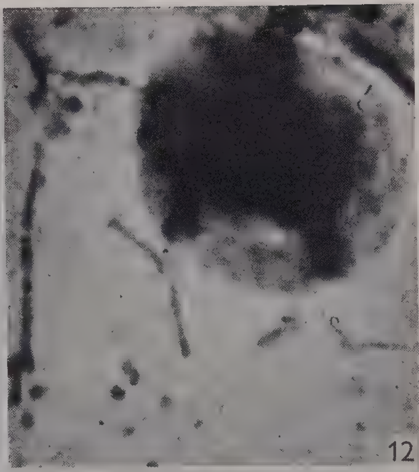
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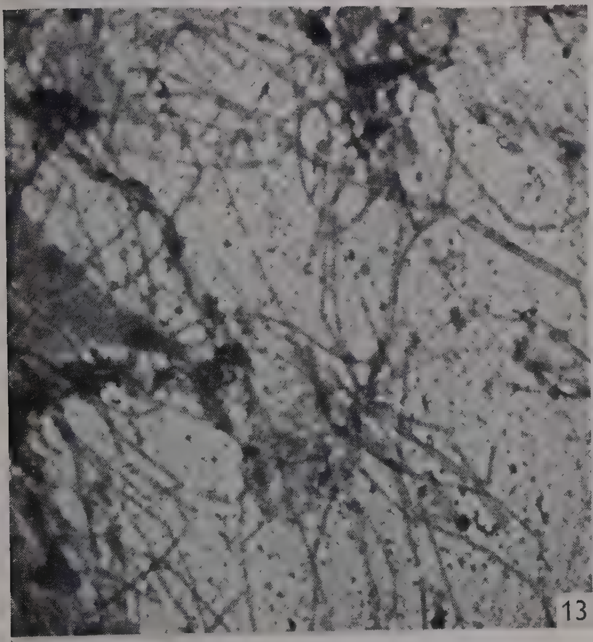
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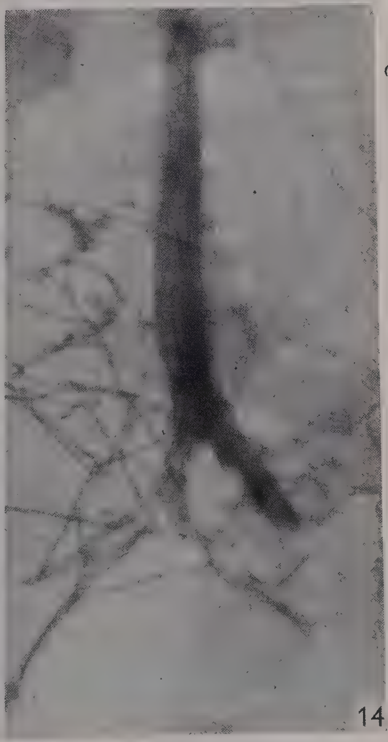
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T. H. Flewett & C. E. Challice—Intracellular growth of fowl-plague virus.
Plate 3

Hewitt, L. F. (1951). *J. gen. Microbiol.* 5, 287–292.

Cell Structure of *Corynebacterium diphtheriae*

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SUMMARY: When examined by phase-contrast microscopy living diphtheria bacilli generally appear fairly transparent. They are sometimes unicellular but are frequently divided into compartments by readily visible cross-septa. In *gravis* and *mitis* strains the cross septa are usually few in number and the compartments long, but in *intermedius* strains there are often many cross-septa, and the organism may resemble a chain of cocci fused together. Club-shaped cells with swellings are seen in untreated living cultures as in fixed and stained preparations of killed bacteria, and occasionally branched forms are encountered. Undivided filaments sometimes occur in magnesium-deficient cultures, whilst in magnesium-rich cultures cells with numerous cross-septa are scanty. The presence of magnesium salts has some protective effect against the growth-inhibition produced by beryllium salts. In cultures containing selenite or tellurite the bacteria become red or black, granules containing selenium and tellurium occur and areas resembling chromatinic structures may be seen.

The recorded morphology of the diphtheria bacillus is based mainly on examination of killed organisms fixed and stained by conventional methods, but recently there has been considerable development of new fixing and staining procedures (Badian, 1933; Piekarski, 1937; Stille, 1937; Robinow, 1942; Klieneberger-Nobel, 1945; Knaysi, 1946; Mudd & Smith, 1950; Smith, 1950). It is of interest to determine how much the appearances seen are artefacts and how they correspond to the morphology of living untreated cells.

The occurrence of club-shaped swellings led Lehmann & Neumann (1899) to use the name *Corynebacterium* which is still generally accepted (Topley & Wilson, 1946; Bergey, 1948), although by no means all of the organisms in diphtheria cultures show swellings when stained. If, as suggested by Bisset (1949, 1950), the swellings are due to artefacts caused by fixing and staining, and if diphtheria bacteria are clearly rod-shaped or coccal in the natural untreated state, some alteration in nomenclature and classification would seem desirable.

Since the microscopy of living cells using phase-contrast microscopy is relatively recent, it is not yet certain what cellular structures apart from external shape will be visible in different preparations, nor what methods of preparation will be most effective.

MATERIALS AND METHODS

The diphtheria strains examined were those serologically typed previously (Hewitt, 1947) and recently isolated strains. The culture media used for their propagation were Loeffler serum, serum agar and blood agar plates, and various special broths which will be mentioned later. Most of the preparations examined were made by modifications of a slide-cell technique (Fleming, Voureka, Kramer & Hughes, 1950; Pulvertaft & Weston, 1950). Delicate organisms may be damaged by subjection to prolonged drying or warming. In order to avoid such damage moist smears of bacteria on cover-slips should not be allowed to remain exposed to air for long and if mounted in melted media, the temperature should be allowed to fall before the culture is covered. The refractive index of 30 % gelatine, as well as its melting-point, make it superior to agar for many purposes, and in most cases good results were obtained with gelatine. All the preparations were examined using the 2 mm. phase-contrast objective of Cooke, Troughton & Simms.

OBSERVATIONS

The cytoplasm of living cells of *C. diphtheriae* in the preparations described above, is often almost transparent, and cross-septa dividing the cells into compartments are plainly visible without staining. In the *gravis* and *mitis* strains depicted in Pl. 1, figs. 1–5, cells containing from one to three cross-septa are seen. Figs. 1 and 2 depict the same field at a 2 hr. interval; in that time an extra cross-septum has appeared in a cell in the centre of the field. Division of the cell occurs at the cross-septum but no previous constriction of the cell at this point has been observed. The two daughter cells suddenly bend apart giving rise to the familiar 'chinese letter' formation. For comparison there are shown in Pl. 1, figs. 6, 7, two preparations of killed diphtheria bacilli which were fixed in osmium tetroxide vapour and 70 % ethanol, mordanted in 10 % tannic acid and stained with crystal violet, a procedure which is used to demonstrate cell walls (Gutstein, 1924). The close similarity between the appearance of living cells examined by phase-contrast microscopy and of the stained preparations is very striking, in contrast to the very different appearance of preparations made by conventional routine heat fixation and staining methods.

Some strains in particular show, in addition to simple almost rod-like forms, club-shapes and swollen-ended organisms (Pl. 1, fig. 3; Pl. 2, figs. 16–19). Occasional indisputably branched forms are encountered, as can be seen clearly in Pl. 2, figs. 8–13. Nuclear structures are not generally visible in cultures grown in ordinary media but some detailed structure may be discerned in Pl. 3, fig. 32.

Intermedius strains are very different in appearance from *gravis* or *mitis* strains. Numerous cross-septa divide the cells into circular or oval coccus-like compartments, each of which can be shown by appropriate staining methods to contain a chromatinic body. In Pl. 2, fig. 14, is shown a phase-contrast photograph of an *intermedius* strain, and in Pl. 2, fig. 15, the same strain fixed in osmium tetroxide vapour and 70 % ethanol, heated with N-HCl and stained with dilute Giemsa solution (Robinow, 1942). It is evident that each cellular compartment is bounded by cell walls and contains a nuclear structure. The typically barred appearance of *intermedius* strains stained by conventional routine methods is evidently due to this multi-cellular structure and the deeper staining of the nuclear material in the separate compartments. In some *intermedius* cultures organisms are seen in which a cross-septum has formed across the widest part of the cellular compartment (Pl. 2, figs. 16, 17).

Diagrammatic representations of typical forms of living diphtheria bacilli are shown in Fig. 1. In *k* are shown the cell walls of an *intermedius* strain and in *l* the nuclear material in the cell compartments as revealed by the Giemsa staining method just mentioned.

Webb (1948) described the effect of magnesium deficiency in inhibiting cell-division of several species of Gram-positive bacteria. In the case of *C. diphtheriae* magnesium deficiency leads to restriction or even complete inhibition of growth, and occasionally filamentous forms are seen. Pl. 3, fig. 29, is a photograph of a *gravis* strain grown in a magnesium-deficient broth. Cross-septa have formed, but final rupture of the cells has not occurred and filaments are formed. In Pl. 3, fig. 30, is shown the same strain grown at the same time in the same broth to which 0·1 % of magnesium sulphate had been added. No cross-septa are visible and the short cells possess more highly refractile areas at the poles, perhaps due to accumulation of magnesium nucleic acid complexes. Another example of filamentous forms of *C. diphtheriae* grown in magnesium-deficient media is seen in Pl. 3, fig. 31. In some *gravis* strains magnesium deficiency leads to the appearance of numerous cross-septa in the cells so that they resemble *intermedius* strains.

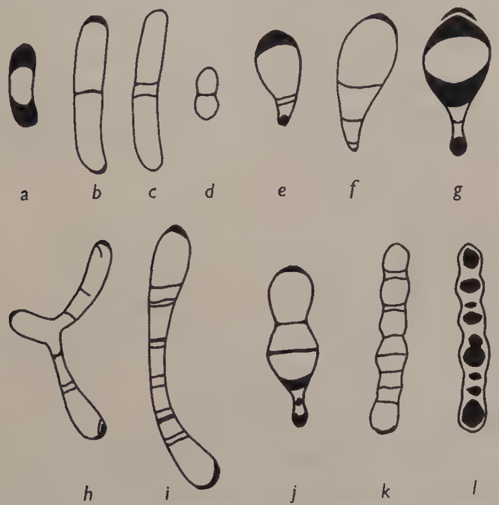

Fig. 1. Typical cell forms of *C. diphtheriae*. (All represent phase-contrast appearances of living cells except *l* which is a stained preparation after fixation with osmic acid and hydrolysis with acid.)

Beryllium salts in a concentration of more than about 0·004 % inhibit the growth of *C. diphtheriae*, some strains being more sensitive than others. In the presence of a large excess of magnesium salts (1 %), this inhibition may be partly reversed, suggesting the possibility that beryllium may be inhibitory to growth by virtue of competitive antagonism to magnesium which is needed for cellular enzymic processes.

The reduction of tellurite by diphtheria bacilli is well known (Conradi & Troch, 1912), and in media containing selenite colonies of *C. diphtheriae* become scarlet-red in colour owing to the production of selenium (Hewitt, 1947). The black or grey cells of *C. diphtheriae* grown in tellurite media possess some unusual forms; the living bacteria appear curved and are difficult to photograph. Highly refractile cellular structures are seen and frequently there are pairs of small spots in each cellular compartment (Pl. 2, figs. 20–25). The spots are of high refractive index and sometimes appear light by phase-contrast microscopy owing to their very highly refractile nature. Some of the grotesque forms seen are represented diagrammatically in Fig. 2. Diphtheria bacilli grown in selenite media also have highly refractile patches as seen in Pl. 3, figs. 26–28. It seems that the elementary tellurium and selenium when formed by cellular reduction processes may be deposited locally in the cell, possibly in the region of nuclear structures.

The appearance of living cells of a few different corynebacteria are shown in Pl. 3, figs. 33–38. Included are *C. pyogenes*, *C. hofmannii*, a diphtheroid, and *C. fascians*, a plant infective agent (kindly supplied by Dr S. E. Jacobs).

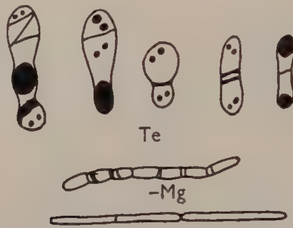

Fig. 2. *C. diphtheriae*; living cells grown in media containing tellurite or selenite, and in magnesium-deficient broth.

DISCUSSION

The pleomorphism of *C. diphtheriae* is very obvious when living cells are examined by phase-contrast microscopy. Small oval bacillary forms, long segmented bacteria and occasionally branched forms are seen. Organisms with club-shapes and polar swellings occur and justify their nomenclature as corynebacteria. Since the club-shapes are seen in living cultures they are not artefacts due to fixing and staining. In many culture media the relatively uniform refractivity and transparency of the cells make easily visible the cross-septa which are frequently present and divide the cells into two or more compartments. In the case of *mitis* and *gravis* strains there are generally few cross-septa and the compartments are long, but in *intermedius* types there are numerous almost circular compartments. This segmentation accounts for the characteristic barred appearance of *intermedius* strains, since the cells contain many cross-septa and they often resemble a chain of fused cocci. The external shape is a series of bulges, each bulge representing a single cellular compartment which by appropriate fixing and staining methods can be shown to contain chromatinic material. Presumably each cellular compartment on segmentation contains a complete genetic structure for the transmission of the characteristics of the strain. Although similar in fermentation reactions, *intermedius* strains can be differentiated from those of the *mitis* group, not only by their appearance when stained but also by serological methods (Hewitt, 1947). The segmented appearance of the living cells provides one more piece of evidence that the differentiation into *intermedius* and *mitis* groups is firmly based on definite cytological differences.

Shrinkage during heat-fixation alters considerably the appearance of *C. diphtheriae* stained by routine methods, but there is a very close similarity in the appearance of the cell walls and cross-septa in living cells and in stained preparations fixed by osmic acid and mordanted with tannic acid.

A most important factor in the morphology of bacteria is the composition of the culture medium. Normally multiplication of *C. diphtheriae* occurs by division at the position of the cross-septa, the daughter cells bending apart, ultimately forming a complex group of adjacent cells which readily entrain other organisms, rendering single-cell culture and isolation very difficult. As reported by Webb (1948) in the case of other organisms, magnesium deficiency in the culture medium interferes with cell division of *C. diphtheriae* and undivided bacteria with many cross-septa may be found. Some evidence was obtained of mutual antagonism between magnesium and beryllium salts and some reversal of the toxic effect of beryllium by a large excess of magnesium salts was observed.

The black and red colour assumed by diphtheria bacilli grown in tellurite and selenite media respectively is due to deposition of the elements in defined localities in the cells and there appears to be replication of these deposits in each segment of the cell. It appears possible that these depositions occur at the site of the nuclear structures of the cell compartments and the pairs of refractile spots seen are similar to the pairs of chromatinic structures reported by Robinow (1942) in spores of *B. mycoides*. Further investigation on these points is necessary, but it seems certain that phase-contrast microscopy, combined with variation in culture media and with other chemical treatments, is likely to contribute considerably in the development of bacterial cytology.

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EXPLANATION OF PLATES

All the photographs are of living cells of *C. diphtheriae* examined by phase-contrast microscopy unless otherwise stated.

PLATE 1

Figs. 1, 2. Same field at 2 hr. interval showing formation of new cross-septum in central cell in Fig. 2; ×3500.

Fig. 3. Long forms; ×3500.

Figs. 4, 5. Transparent cells showing cross-septa; ×3500.

Figs. 6, 7. Stained preparations. Fixed with osmic acid and 70 % ethanol, treated with 10 % tannic acid for 30 min. and stained with crystal violet; ×3500. Note similarity to living cells in other figures.

Figs. 8–13. Branched forms; ×3500.

PLATE 2

Fig. 14. *Intermedius* strain showing multiple cross-septa; ×3500.

Fig. 15. *Intermedius* strain stained with Giemsa stain after fixation with osmic acid and ethanol and hydrolysis with N-HCl showing chromatinic structures in segments of cells between cross-septa; ×3500.

Figs. 16, 17. 'Violin' shapes with cross-septa across wide part of cells; ×1800.

Figs. 18, 19. Swollen and club forms; ×1800.

Figs. 20–25. Living cells grown on tellurite-blood agar plates showing tellurium granules and cellular structure; ×3500.

Figs. 26–28. Living cells grown on selenite-blood agar plates showing cellular structure; ×3500.

PLATE 3

Fig. 29. Cells grown in magnesium-deficient broth showing filaments; ×3500.

Fig. 30. Same strain as Fig. 29 and grown in same broth with 0·1 % $MgSO_4$ added. Note short bacteria with polar refractile material but no cross-septa; ×3500.

Fig. 31. *Gravis* strain from magnesium-deficient broth showing filament with cross-septa; ×3500.

Fig. 32. Internal structure visible in long form; ×3500.

Figs. 33, 36. *C. hofmannii* (*C. pseudodiphtheriticum*); ×3500.

Fig. 34. *C. pyogenes*; ×3500.

Fig. 35. A diphtheroid; ×3500.

Figs. 37, 38. *C. fascians*; ×3500.

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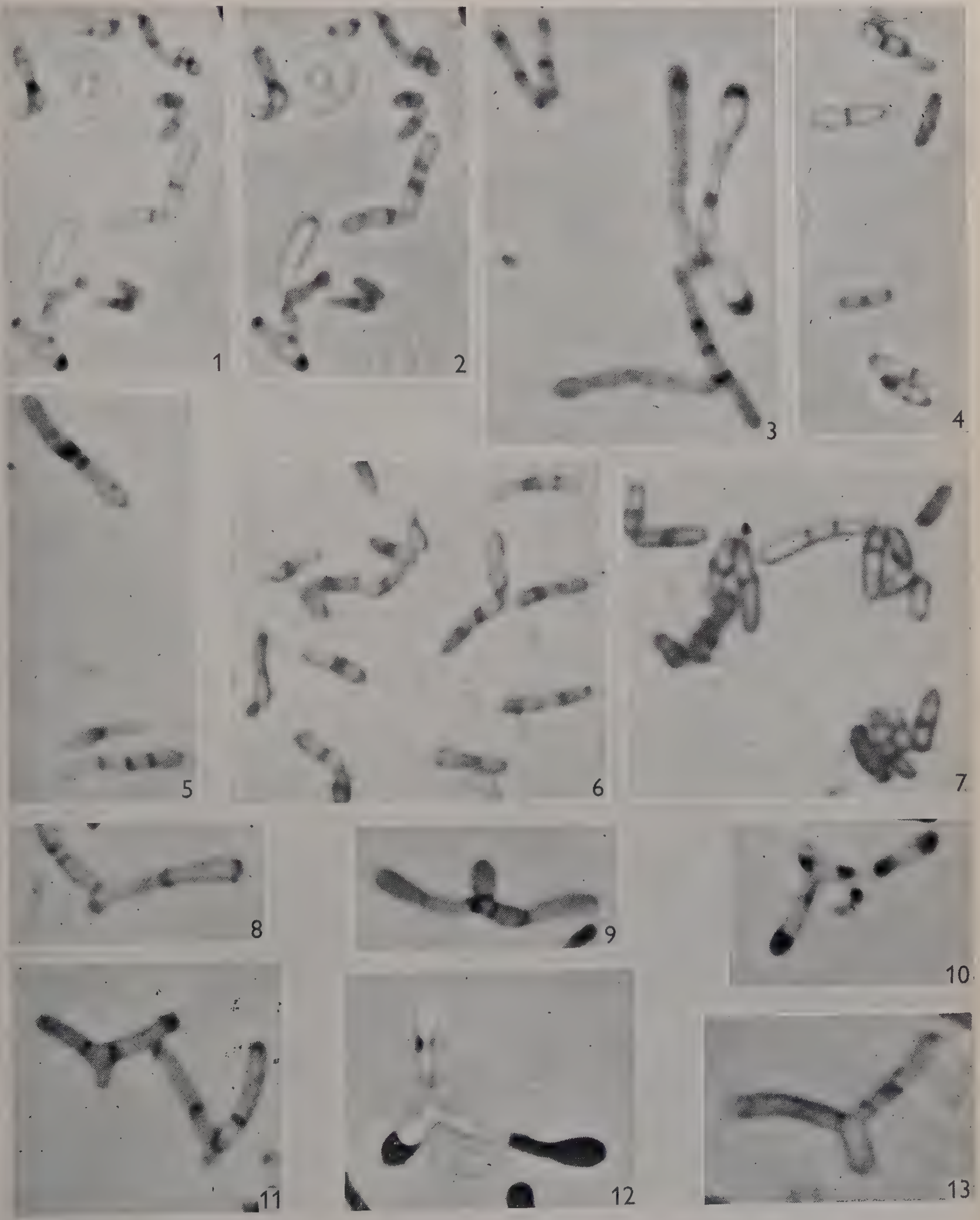
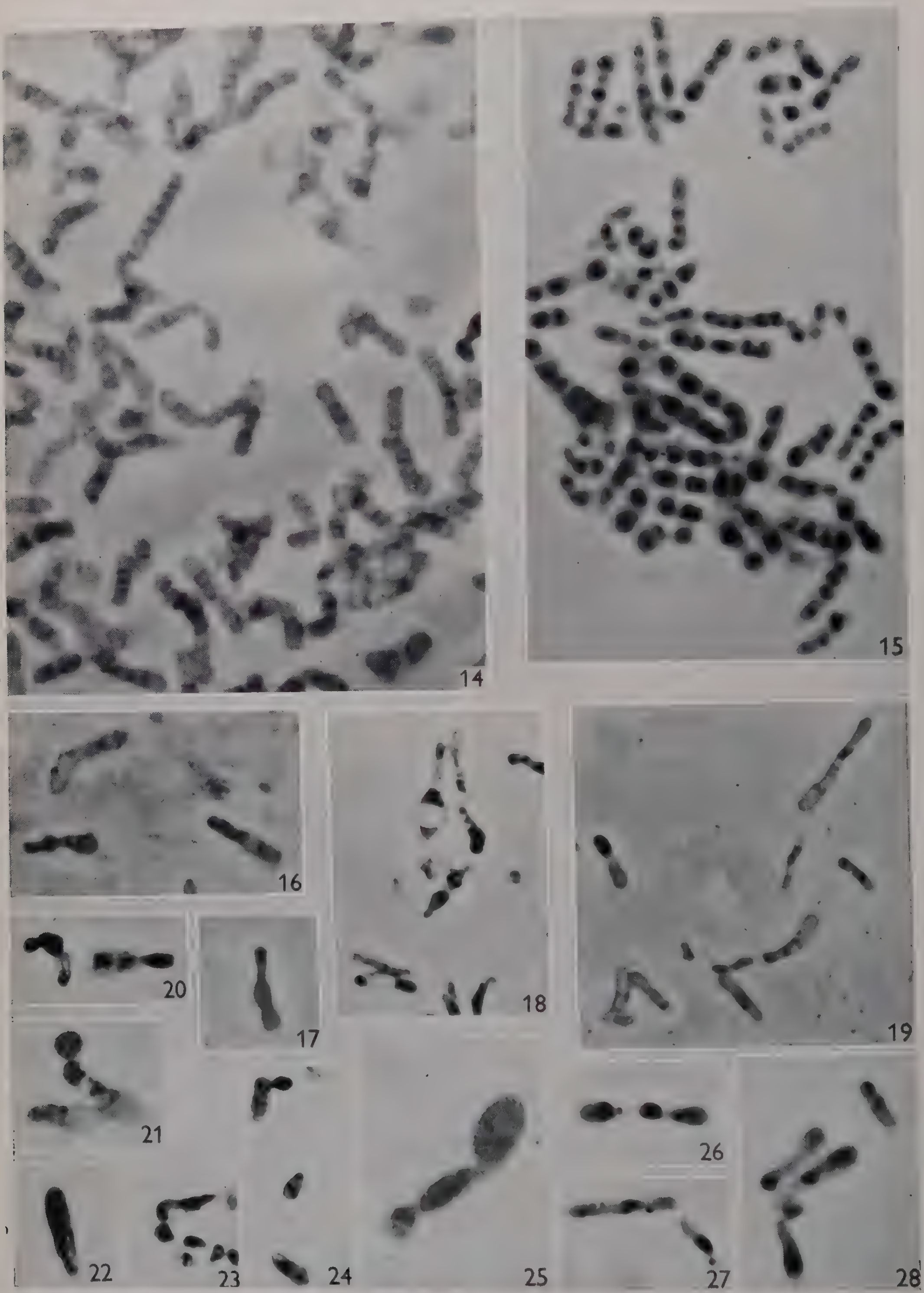
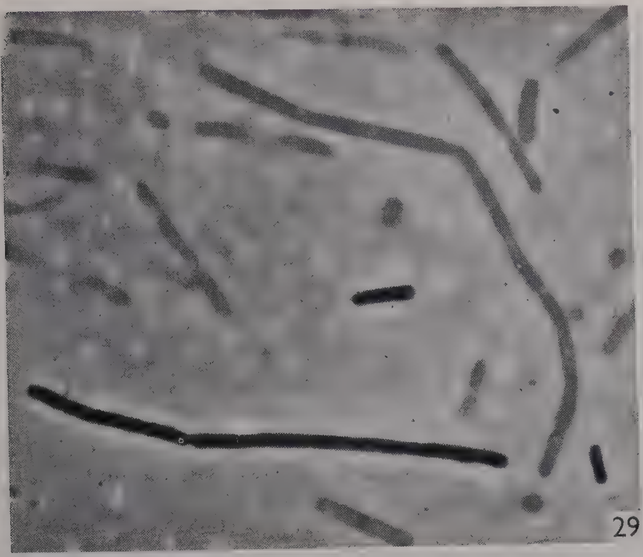


L. F. Hewitt—Cell structure of *C. diphtheriae*. Plate 1



L. F. Hewitt—Cell structure of *C. diphtheriae*. Plate 2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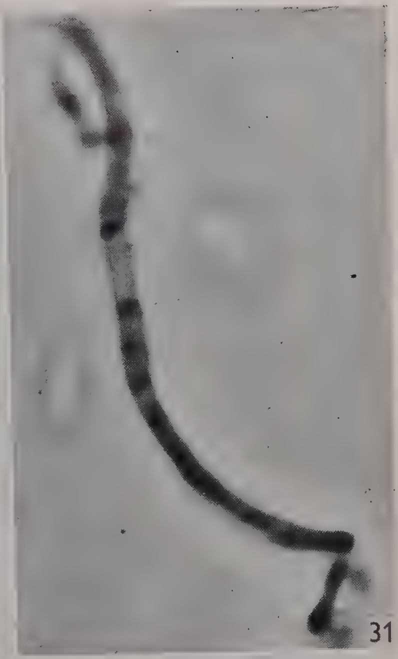
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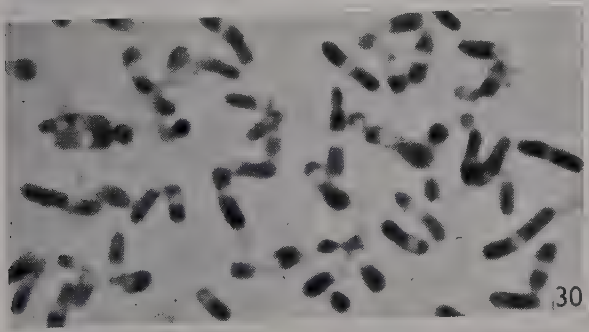
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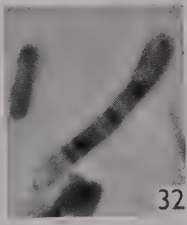
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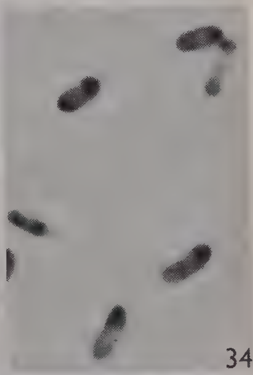
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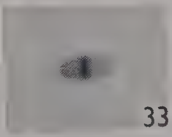
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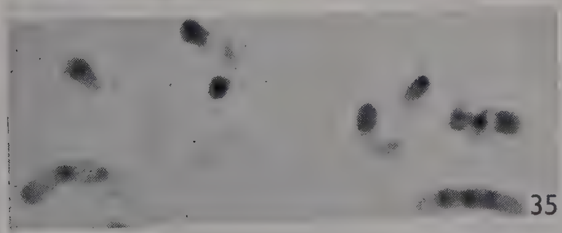
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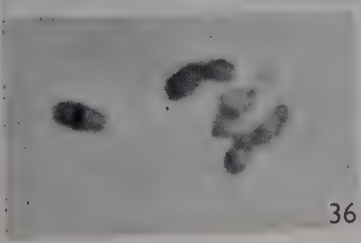
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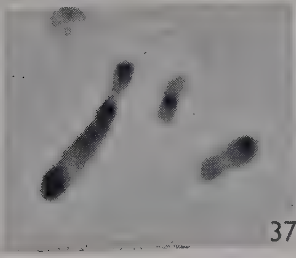
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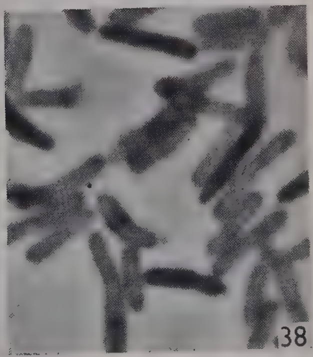
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L. F. HEWITT—CELL STRUCTURE OF *C. DIPHTHERIAE*. PLATE 3

HEWITT, L. F. (1951). *J. gen. Microbiol.* 5, 293–297.

Effect of Cultural Conditions on Bacterial Cytology

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SUMMARY: Phase-contrast microscopy of living bacterial cells under appropriate conditions is of value in determining cellular structure, bacterial nuclear processes, methods of division and the effects of cultural conditions. Different species of bacteria differ in their methods of division (for example by constriction or by cross-septation) and in their apparent internal structure. Considerable differences may be seen also in the same organism grown under different cultural conditions. The same species of bacteria may exist as short oval bacilli in an agar medium and long-chained or filamentous forms in broth, with equally characteristic differences in the distribution of refractile material within the cells. The incorporation of special chemical compounds in the culture medium raises the possibility of vital 'staining' by the deposition of refractile material in defined sites within the bacteria during growth, thus rendering visible by phase-contrast microscopy certain cellular structures in living bacteria. The inclusion in culture media of tellurite and selenite has this effect of showing up internal structure in the bacteria grown in their presence. Apart from the possibility of developing new procedures for detecting internal structure, phase-contrast microscopy is of use in evaluating fixation and staining methods by comparing the fixed and stained specimens with living cells, and thus recognizing distortions and artefacts introduced by preparative treatments.

Apart from its purely descriptive value for classification and diagnosis bacterial morphology is being used increasingly for the study of cell structure and physiology. Although this is no recent development (Schaudinn, 1902; Dobell, 1911), advances in technical methods such as those of Badian (1933), Stille (1937) and Piekarski (1937) have led Robinow (1942), Klieneberger-Nobel (1945), and Bisset (1950) to formulate very definite concepts of bacterial structure. The newer methods differ in two principal respects from the older ones which are still used for routine purposes. First, fixation of the organisms is effected, not by drastic heating methods which are likely to cause distortion and shrinkage of the cells, but by gentler methods less likely to cause damage. Secondly, new chemical procedures are introduced into the staining technique in order to demonstrate specific cytological structures such as cell walls and nuclei. Nevertheless, the procedures remain very drastic and include exposure to osmic acid vapour, treatment with cold tannic acid and hot hydrochloric acid, etc. Hence it remains desirable to examine living untreated cells in order to evaluate new procedures and to determine what appearances are in fact vital and which are artefacts. Unfortunately, the electron microscope, which might be of value for this purpose, has several limitations; completely dry specimens must be examined, and because protoplasm is so opaque to electrons chemical treatment is often necessary to reveal many details of internal cellular structure.

Phase-contrast microscopy does not suffer from these particular limitations, moist living cells may be examined under physiological conditions, and it would be anticipated that differences in refractivity might reveal some details of internal cell structure. In the case of *Corynebacterium diphtheriae* (Hewitt,

1951) the cytoplasm under suitable conditions appears quite transparent and some details of structure, especially cell walls, are visible, and differences in cultural conditions are reflected in variations in appearance, but a considerable amount of work is required before it can be decided what structures are likely to be visible in the phase-contrast microscope, which techniques are likely to be most useful and whether any developments of methods are likely to be fruitful. Not the least point of value of the method is the possibility of comparing the appearances of living cells with those observed in preparations made by both the older and newer fixing and staining procedures. This communication, therefore, is largely an exploratory one aimed at seeking possible lines of advance and reporting the results obtained so far.

MATERIALS AND METHODS

The organisms, mostly isolated in the laboratory, were grown in a variety of liquid and solid media of which the composition was varied as described later. Preparations for microscopy were made by modifications of the cover-slip cell method (Hewitt, 1951) and examined with a 2 mm. Cooke, Troughton & Simms phase-contrast objective.

OBSERVATIONS

In Pl. 1, figs. 3–5, is seen a great variety of forms of *Bacillus cereus* which had been grown on a broth agar plate. Some cells are almost uniformly opaque, others contain both opaque and clear patches, whilst still others are almost completely transparent except for the cell walls and opaque roughly circular inclusions. These circular granules occur frequently in triplets or sextets, and a series may be arranged as in Fig. 1. It is easily possible to postulate that these are nuclear structures with a unit of three chromosomes to each cell and their re-arrangements resemble mitosis. This method of reconstruction is, however, very hazardous and there is no supporting evidence that these granules have any nuclear significance, since cells seen to divide whilst under observation usually have a more refractile appearance. Rearrangement of the dark granules may be observed and the transparent cells may be seen to change rapidly into the refractile type. Sometimes also the refractile cells become transparent with dark granules. Nuclear material in bacterial cells can be demonstrated by Giemsa staining after fixation with osmic acid vapour and hydrolysis with hydrochloric acid (Robinow, 1942), but when *Bacillus cereus* is treated in this way the dark granules are not stained. This may be seen in Pl. 1, figs. 6, 7. The cells were stained by this method and then examined by phase-contrast and by ordinary microscopy. The granules which appear dark and highly refractile by phase-contrast are unstained and appear light by ordinary microscopy. It would seem probable that the granules are not nuclear in nature but probably fat-storage depots.

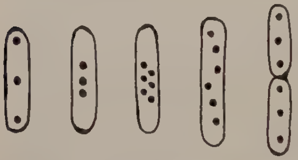


Fig. 1. Refractile granules in *B. cereus*.

As already mentioned, dividing cells of *B. cereus* are usually almost uniformly dark in colour in the phase-contrast microscope but the formation of cross-septa or cell walls can be seen to occur at the point where division afterwards occurs. In Pl. 2, figs. 27–32, are seen serial photographs of a *B. cereus* cover-slip culture at 37° taken at approximately 45 min. intervals. The formation of cross-septa, the 'thinning' of the protoplasm at this point and constriction of the cells preceding division, are visible.

In some cocci refractile material resembling a central nucleus can be distinguished in living untreated cells (Pl. 2, figs. 11–17) as in specimens stained by methods designed to demonstrate presence of nuclear material (Bisset, 1950). In the case of other organisms, however, no nucleus can be seen by phase-contrast microscopy. Refractile material resembling nuclear or chromatinic structures is very prominent in the Gram-positive bacillus shown in Pl. 3, fig. 34.

There is a number of different processes of division among the cocci. In some a cross-septum forms as in the diplococci shown in Pl. 2, fig. 2, and in the yellow tetrad organism in Pl. 2, figs. 18–21. In the latter case cross-septa at right-angles are formed and so give rise to tetrads. With other cocci such as the haemolytic streptococcus in Pl. 2, fig. 26, and the coccus in Pl. 2, figs. 11–17, the cell elongates and a constriction appears before fission but no cross-septa have been seen. Clear observation of *Haemophilus pertussis* is difficult owing to the envelope surrounding the organisms; occasionally a structure resembling a cross-septum may be seen in the cell, but it would appear that fission takes place by constriction of the cell. Some evidence of nuclear structure in the cells may be seen in Pl. 2, figs. 22, 23. In staphylococci cross-septa are formed at right angles to one another (Pl. 2, fig. 25).

The effects of cultural conditions on bacterial morphology are very striking. In Pl. 3, figs. 35, 36, are seen cells of *Pasteurella pseudotuberculosis* isolated from a pancreatic nodule in a guinea-pig and grown in various media. On agar plates (Pl. 3, fig. 35) the organisms are small ovals and almost transparent, whilst in broth (Pl. 3, fig. 36) the organisms are longer, contain refractile material at the poles and they remain joined together in long festoons. Presumably the differentiation of refractile material in broth cultures accounts for the commonly observed polar staining of the organisms. In the case of the Gram-positive bacilli seen in Pl. 2, figs. 33, 34, the agar plate cultures are in the form of separate bacilli whilst in broth long filaments are formed and the organisms contain refractile bodies resembling chromatinic structures.

The conclusions of Webb (1949) that magnesium ions are necessary for the fission of bacteria during multiplication are illustrated in Pl. 1, figs. 1, 2, in which cells of *Bacillus cereus* grown in magnesium-deficient media form long filaments. These filaments contain cross-septa and segments differing in refractive index, some being almost transparent and others deeply refractile but fission of the organisms has not occurred. In Pl. 1, figs. 3–5, are shown the same organism grown in media containing ample magnesium ions and the normal appearance is seen.

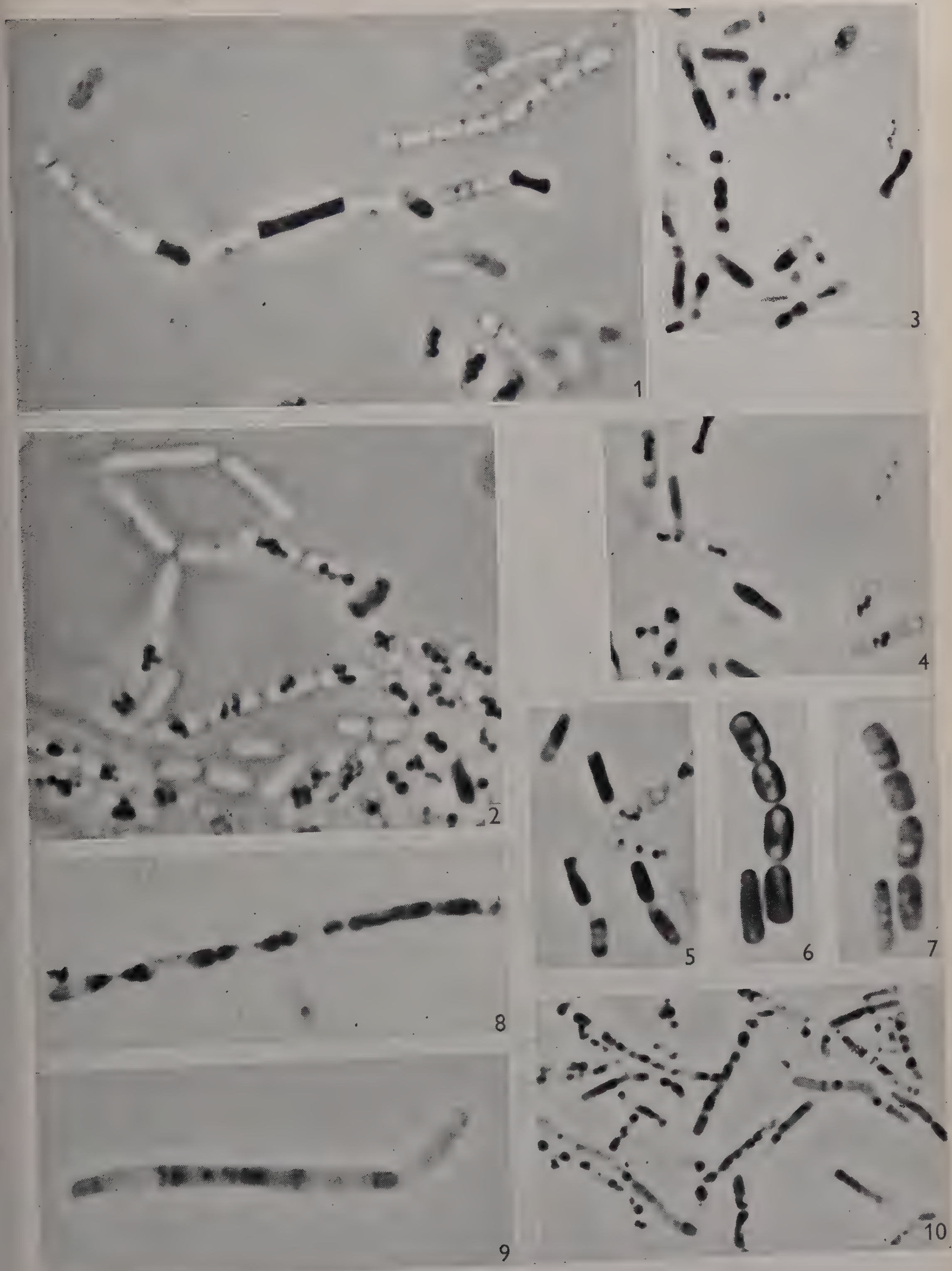
In a previous paper it was shown (Hewitt, 1951) that *Corynebacterium diphtheriae* cultures reduce tellurite and selenite to the elements tellurium and selenium, becoming black or red, and when examined by phase-contrast microscopy the cells are seen to contain granules of the elements and refractile deposits in localized sites in the cells. These localized sites resemble nuclear structures and this method of growing organisms in media containing reducible substances which are deposited locally in the cell may constitute a useful means of recognizing nuclei or other cellular structures. In Pl. 1, figs. 8–10, are shown cells of *Bacillus cereus* grown in the presence of selenite. Some filamentous forms are seen containing cross-septa and refractile patches which may represent nuclear structures. *Corynebacterium fascians* grown in the presence of selenite is shown in Pl. 3, fig. 37; again dark refractile patches resembling nuclei can be distinguished. It is possible that nuclear material may have an affinity for tellurite and selenite or may be regions of intense metabolic activity where reduction to the element takes place preferentially.

DISCUSSION

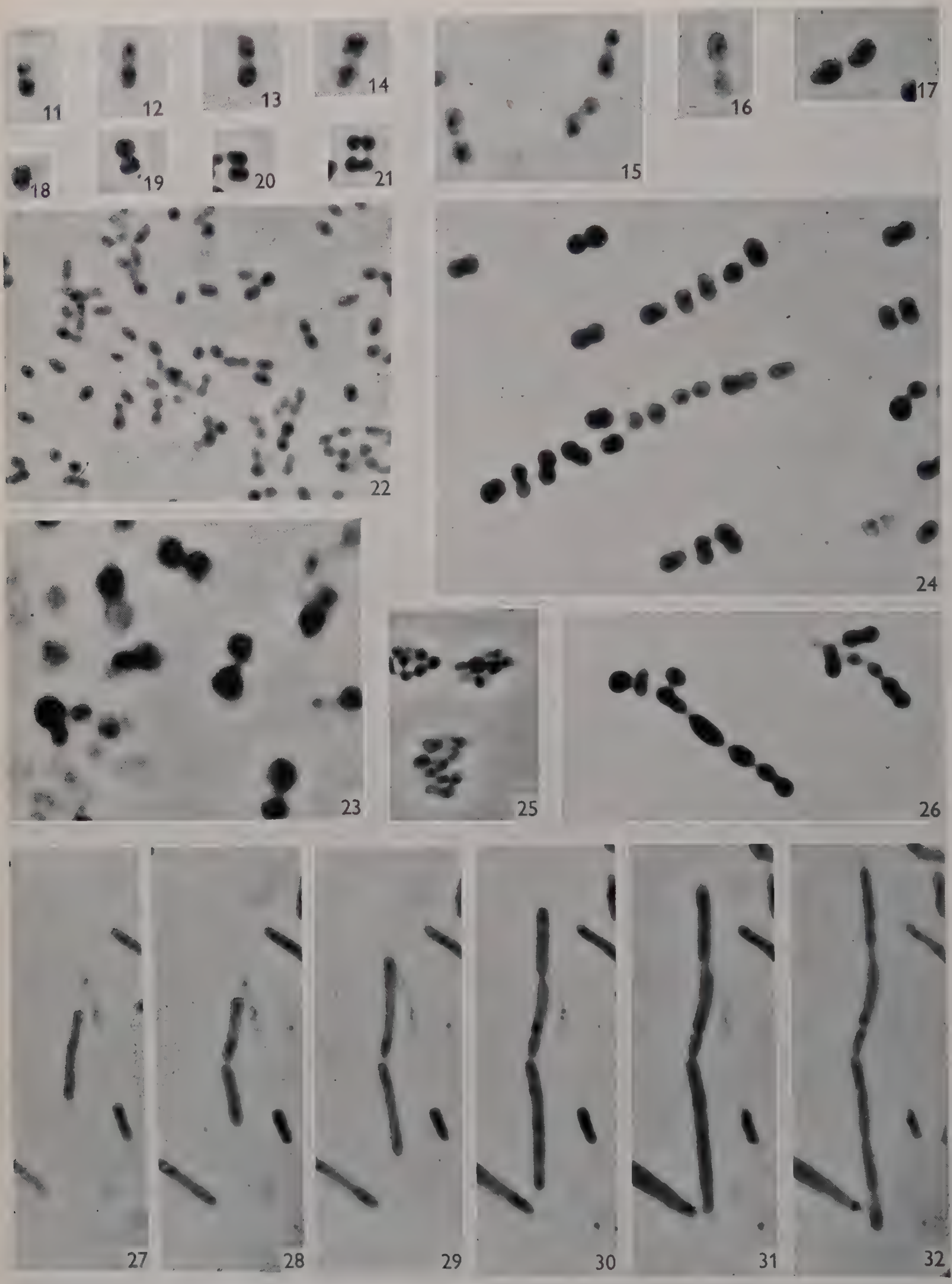
Phase-contrast microscopy of living bacterial cells promises to present new evidence on problems concerning the mechanism of bacterial multiplication and the existence of bacterial nuclei, since possible distortions and artefacts due to fixation and staining procedures are avoided, and actual division of cells may be observed so that speculative reconstruction is unnecessary. It is not yet certain however what cellular structures can be discerned by phase-contrast examination, nor what cultural or preparative method may be effective in demonstrating ordinarily transparent structures.

Differences in the mechanism of fission of the daughter cells during bacterial multiplication (Robinow, 1942; Bisset, 1950) can be seen clearly by phase-contrast methods. Division may occur as in the case of *C. diphtheriae*, by the formation of an easily visible cross-septum and by the daughter-cells bending apart. With other organisms a double cell-wall appears to develop at the point of fission, a constriction appears in the mother-cell at this site, and the daughter cells may remain attached in a chain-like formation. The relative alinement of successive cross-septa accounts for the different groupings of cocci in pairs, tetrads, clusters, etc. When successive cross-septa are parallel, chains may be formed, whilst if they are at right angles tetrads or cubes occur. In magnesium-deficient media (Webb, 1949) cross-septum formation may be normal but actual fission of the cell is inhibited and long filaments may occur. It is possible that magnesium ions constitute an essential co-enzyme for the final fission process. Other cultural variations also affect the arrangement and appearance of bacterial cells. Frequently when grown on agar plates bacteria may appear as single transparent cells, whilst in broth they may occur as festoons of bacteria containing differentiated refractile patches in the cells.

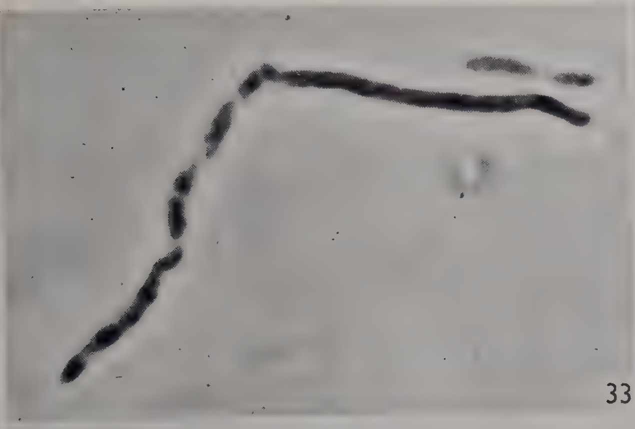
In some culture media many bacteria appear uniformly transparent by phase-contrast microscopy except for cell walls and food-storage granules, and the latter may be mistaken for nuclear processes, but other organisms appear to possess structures which may well be nuclear, and the incorporation of various chemical substances (such as selenite) in the culture medium may render visible in living cells transparent structures by the localized deposition of refractile material.



L. F. Hewitt—Bacterial cytology. Plate 1



L. F. Hewitt—Bacterial cytology. Plate 2



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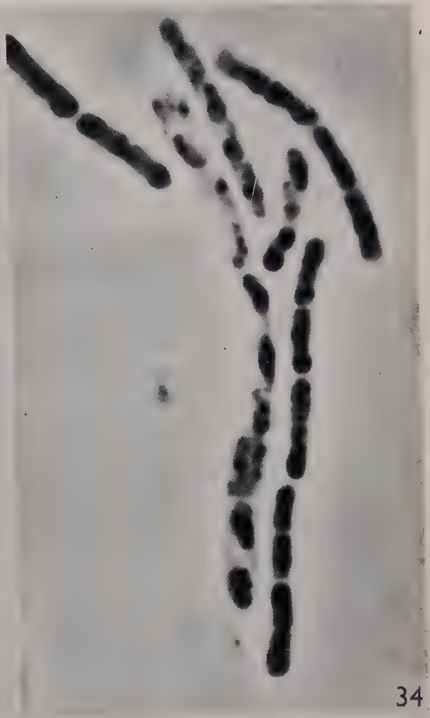

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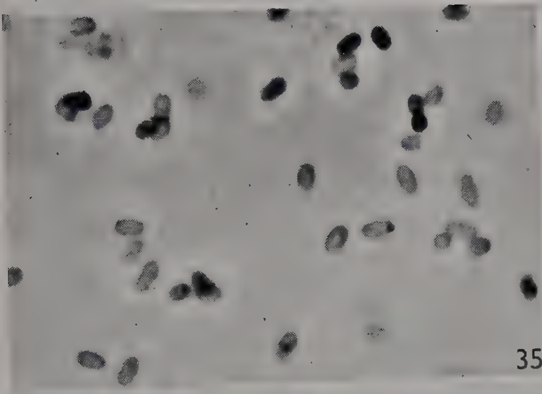

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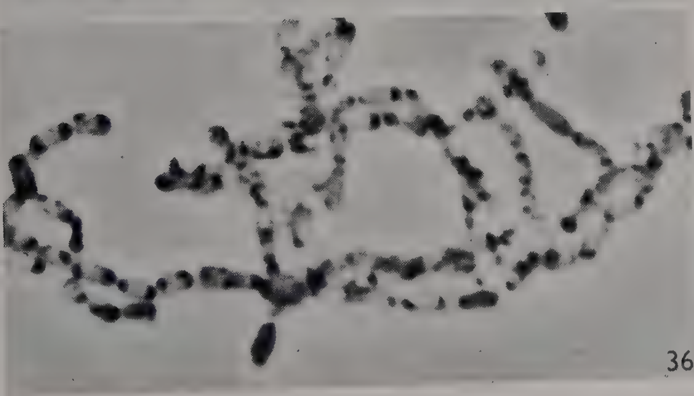

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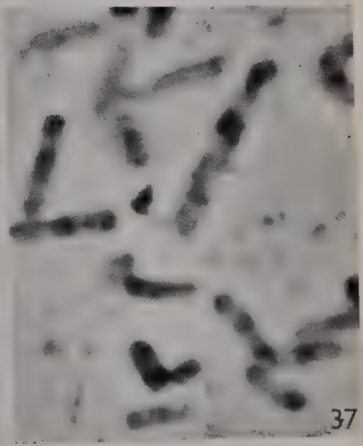

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L. F. HEWITT—BACTERIAL CYTOLOGY. PLATE 3

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EXPLANATION OF PLATES

(All are phase-contrast photographs of living cells except figs. 6 and 7.)

Plate 1

Figs. 1, 2. *Bacillus cereus* from magnesium-deficient media; note filaments and abundance of transparent cells; ×3500.

Figs. 3–5. *B. cereus* grown with adequate magnesium content in medium; no filaments, note arrangements of spherical refractile bodies; ×3500.

Figs. 6, 7. *B. cereus*, fixed osmic acid, hydrolysed with hydrochloric acid and stained with Giemsa. Fig. 6 is a phase-contrast photograph and fig. 7 with ordinary microscope. Dark granules in phase-contrast appear light and unstained in ordinary microscope; ×4000.

Figs. 8–10. *B. cereus* grown in selenite medium. Figs. 8 and 9, ×3500. Fig. 10, ×2000.

Plate 2

Figs. 11–17. Mode of division of a Gram-positive coccus. Note central refractile body; ×3500.

Figs. 18–21. Mode of division in tetrads, note cross-septa; ×3500.

Figs. 22–23. *Haemophilus pertussis*; ×3500.

Fig. 24. Mode of division of a diplococcus; ×3500.

Fig. 25. *Staphylococcus*; ×3000.

Fig. 26. Haemolytic *Streptococcus*; ×3500.

Figs. 27–32. Serial photographs showing multiplication of *Bacillus cereus*; ×3500.

Plate 3

Figs. 33–34. Gram-positive bacillus. Fig. 33 from broth, fig. 34 from agar plate. Highly refractile structures in cells; ×3500.

Figs. 35–36. *Pasteurella pseudotuberculosis*. Fig. 35 from agar plate, fig. 36 from broth; ×3500.

Fig. 37. *Corynebacterium fascians* from selenite medium; ×3500.

(*Received* 16 *June* 1950)

Miles, E. M. & Miles, A. A. (1951). *J. gen. Microbiol.* 5, 298–306.

The Identity of *Proteus hydrophilus* Bergey *et al.* and *Proteus melanovogenes* Miles & Halnan, and their Relation to the Genus *Aeromonas* Kluyver & van Niel

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SUMMARY: *Proteus melanovogenes*, originally isolated from black-rot in eggs, does not differ from *Pr. hydrophilus*, the cause of red-leg in frogs, more than different strains in each species differ from one another; and the two species may be considered as identical.

Both species resemble *Aerobacter liquefaciens* Beijerinck, *Pseudomonas fermentans* von Wohlzogen Kühr and *Ps. ichthyosmia*. The divergences from a common type displayed by these 'species' may be sufficient to warrant the preservation of some as separate species, some as varieties, but all strains of this type with the property of producing 2:3-butanediol from glucose should be allotted to the genus *Aeromonas* in the Pseudomonadaceae, as recommended by Kluyver & van Niel (1936).

In 1937, Miles & Halnan isolated a monotrichate, Gram-negative bacillus which was the cause of black-rot in South African eggs imported into England, and which they named *Proteus melanovogenes*. They found a similar bacillus in English soils and manures, and in the faeces of a patient with chronic colitis. Aitken, Barling & Miles (1936) also found a similar bacillus (Strain M90) in a meat-pie responsible for a fatal *Clostridium botulinum* intoxication in man. From this limited experience it appeared that *Proteus melanovogenes* might be widely distributed and was potentially parasitic in a number of ways.

Miles & Halnan (1937) noted the close resemblance of their bacillus to *Pr. hydrophilus*, the cause of red-leg in frogs, as described by Bergey (1934), but in their view *Pr. hydrophilus* was decisively distinguished by its reported possession of peritrichate flagella, and they accordingly created a new species in the genus *Proteus*. Bergey's description, which they omitted to check, was however incorrect. Of the authorities cited in the 1934 (4th) edition of *Bergey's Manual*, none mentions the number of flagella, though all agree in describing active motility (Ernst, 1890; Sanarelli, 1891; Chester, 1897; Weldin, 1927). Nevertheless, in 1898 Russell had described the bacillus of red-leg as monotrichate. The 1948 edition of *Bergey's Manual* correctly records *Pr. hydrophilus* as monotrichate, and presumably for this reason, transfers the species to the genus *Pseudomonas*. Russell's (1898) and Kulp & Borden's (1942) establishment of *Pr. hydrophilus* as monotrichate clearly indicated a reconsideration of the validity of *Proteus melanovogenes* as a separate species.

SOURCE OF THE STRAINS

Unfortunately most of the original strains of *Pr. melanovogenes* were lost during the 1939–45 war, including the type culture ER1. Two strains survived, ER75 from a rotten South African egg, and M90 from the poisoned meat-pie mentioned above. Since, however, both strains had been proved capable of producing typical black-rot in hen eggs soon after isolation, they may be considered sufficiently representative of *Pr. melanovogenes.* For comparison, we obtained from the National Collection of Type Cultures, two strains of *Pr. hydrophilus* N.C.T.C. 7810 (=American Type Culture Collection 9071; Kulp & Borden's 1942, strain P2) and N.C.T.C. 7812 (=A.T.C.C. 7965; from M.L. Speck); and a strain of *Pseudomonas icthyosmia* (N.C.T.C. 8049=A.T.C.C. 7966). From Prof. Kluyver at Delft we obtained: an authentic strain of *Ps. fermentans* isolated by von Wohlzogen Kühr and now named *Aeromonas liquefaciens* (L 417); a strain of *Aeromonas liquefaciens* (L 418) isolated at Delft; one strain of *A. hydrophila* (L 447) sent to Delft by Dr Hitchner. In June 1949, during an epidemic of red-leg in a dealer's batch of *Rana temporaria*, we also isolated four strains from three frogs—DF 1*a* and DF 1*b* from one frog, DF 2 and DF 3. Nine of the twelve strains produced a rapidly fatal bacteraemia in frogs when injected either intraperitoneally or into the lymph sac in doses of 0·4 ml. of an overnight culture in heart extract broth, the exceptions being the strains *Pseudomonas icthyosmia*, *Ps. fermentans* and *Aeromonas liquefaciens* (L 418). *Pseudomonas icthyosmia*, which was originally isolated from a tin of milk with a fishy smell, has never been associated with red-leg in any way, and appears to be quite harmless to frogs, although pathogenic for mice. The *Ps. fermentans* produced a severe, haemorrhagic local lesion in the thigh of one of three frogs injected subcutaneously and was isolated in pure culture by direct plating of the heart blood of the animal; the other frogs survived 3 weeks in apparent good health. The *Aeromonas liquefaciens* (L 418) had no effect on frogs, little virulence for mice, and differed in other respects from the rest of the group.

All twelve strains induced a characteristic black-rot in eggs in 9–21 days at 37°, when injected by the method of Miles & Halnan (1937), but ER75, M90 and *A. liquefaciens* L 418 were only feebly saprogenic, though the first two when originally isolated had produced severe black-rot. An attempt to enhance saprogenicity by frog and mouse passage was unsuccessful. L 418 produced a true black-rot only in one of the three eggs inoculated. We were clearly dealing with a group of strains generally similar in their parasitizing capacity.

COMPARISON OF THE TWELVE STRAINS

The following description applies to the twelve strains; divergences are noted in brackets.

Morphology. Gram-negative rods, 0·4–0·8 × 1·5–3·0μ., with parallel sides and rounded ends. Non-acid fast. No endospores. Filaments common in 'rough' colony forms. Actively motile by a single polar flagellum about 5–6μ. long (cells of ER75 and DF 3 were either monotrichate, or lophotrichate with two to eight flagella).

Colonial appearance. After 24 hr. on horse blood agar plates, colonies were 2–3 mm. in diameter (L 418, 0·5 mm.), round, entire-edged and raised, smooth moist surface (L417 slightly granular), semi-translucent greyish white in colour (DF 1*b* had opaque colonies and was originally distinguished from DF 1*a* on this ground; both DF1*a* and DF1*b* bred true with respect to colony form: L418 was also opaque); haemolysis marked (ER75 moderately haemolytic, L418 non-haemolytic). After 3–5 days at room temperature a dirty brown-yellow coloration appeared.

Growth in broth. After 24 hr. abundant, turbid, with a moderate, readily disintegrable sediment and fine pellicle.

Gelatin stab. Growth good, liquefaction marked and commonly saccate.

Loeffler's serum slope. Growth abundant (for digestion, see Table 1).

Potato slope. Growth abundant, moist and glistening. Six strains produced salmon pink pigment. The rest varied from creamy white to browny yellow (see Table 1).

Metabolism. Aerobic. Anaerobic growth feeble. Grow well on ordinary laboratory media at 20°, luxuriantly at 37°. In a basal medium containing 1 % glucose and 0·1 % K_2HPO_4 in tap water, utilize 0·1 % $(NH_4)_2SO_4$, uric acid or asparagine as source of nitrogen. In basal medium containing 0·1 % $(HN_4)_2SO_4$ and 0·1 % K_2HPO_4 utilize 0·5 % citrate, but not 0·5 % *meso*-tartrate as source of carbon (*Pseudomonas icthyosmia*, N.C.T.C. 8049, and L418 negative). (For utilization of acetate, lactate and malate, see Table 1.)

Biochemical. Glucose, galactose, fructose, mannose, maltose, sucrose, mannitol, glycerol and starch fermented at 37° in 1–2 days with production of acid and gas. Arabinose, raffinose, rhamnose, dulcitol and inositol not fermented. (L418 ferments both raffinose and inositol with the production of acid only. It also gives a trace of acid in the Durham's tubes with inulin and arabinose.)

The fermentation of salicin is interesting. At 37° only DF3 and ER75 ferment it. Hammer (1917) observed that *Ps. icthyosmia* produced more acid from sugars at 22° than at 37°, and that salicin, notably, was fermented at 22° but not at 37°. With two exceptions (N.C.T.C. 7810 and *Ps. fermentans*, L417) all our strains fermented salicin at 22°. We also tested the equivocal lactose fermentation at this temperature, but the results were no better than at 37° for other sugars; see Table 1).

Litmus milk acidified in one day, then clotted and digested. Nitrates reduced to nitrites. Thiosulphate reduced. Indole positive; H_2S, NH_3, catalase, diastase and methylene-blue reductase positive. Urease negative. (For methyl red, Voges-Proskauer and Koser's citrate tests, and lipase production, see Table 1.)

The formation of 2:3-butanediol from glucose was tested in 3-day cultures in the medium recommended by Stanier & Adams (1944), which was extracted with ethyl ether, and residue after evaporation of the ether tested for acetoin and 2:3-butanediol as recommended by these authors. A strain was regarded as positive if the 2:3-butanediol produced was >4 % of the starting weight of the glucose. All the twelve strains were positive in this sense. The strains of *Ps. icthyosmia*, ER75 and DF1*a* were the least active.

Serological. Antigenically heterogeneous by a limited analysis with agglutinating antisera to all but the DF strains and L447 (Table 2). *Proteus hydrophilus* N.C.T.C. 7810 and 7812 were slightly related, and M90 and N.C.T.C. 7812 closely related. L417 *Pseudomonas fermentans* was markedly related to 7810 and DF3, and slightly to all the rest of the group excepting *Ps. icthyosmia.*

Table 1. *Divergent characters of the twelve strains*

	N.C.T.C. 7810	N.C.T.C. 7812	ER75	M90	DF1*a*	DF1*b**	DF2	DF3	N.C.T.C. 8049	L417	L418*	L447
Flagella	m	m	m+l	m	m	m	m	m+l	m	m	m	m
Fermentation of												
Xylose	–	–	A†	–	–	A	–	–	–	–	A	–
Raffinose	–	–	–	–	–	–	–	–	–	–	A	–
Lactose	a_4	a_4	a_4	a_4	Ag	Ag†	a_4	Ag	a_4	A_6	A_{14}	–
Inositol	–	–	–	–	–	–	–	–	–	–	A_2	–
Sorbitol	–	A	A	A	A	–	A	–	–	–	A	–
Aesculin‡	–	–	A	–	A	–	–	a	nt	nt	nt	nt
Salicin (22°)	–	Ag	AG	Ag	Ag	AG	AG	AG	a	–	a	AG
Salicin (37°)	–	–	AG	–	–	–	–	AG	–	–	–	–
Glycogen and dextrin	A	A	–	A	A	A	A	A	A	A	–	A
Utilization of												
Koser's citrate	–	–	+	+	+	–	–	+	+	+	+	–
Citrate } with (NH$_4$)$_2$SO$_4$	+	+	+	+	+	+	+	+	–	+	–	+
Acetate	–	–	–	–	–	–	+	–	–	–	–	–
Malate	+	+	+	+	–	+	–	+	+	+	+	+
Lactate	–	–	–	–	+	+	–	+	+	+	+	+
Lipase	tr	tr	tr	+	±	±	–	tr	±	±	–	+
Digestion of Loeffler's serum	+	–	+	+	+	–	+	+	+	+	–	–
Voges-Proskauer	+	+	+	+	+	+	+	+	+	+	?	?
Methyl red	–	–	–	–	–	+	–	–	–	+	–	–
Pigment on potato	SP	SP	GB	SP	C	C	SP	SP	CL	CL	CL	C/SP

m, l = monotrichate, lophotrichate.
a, A = slight, marked acidity. } Without suffixed number = 1 day's incubation; suffixed number = day of first appearance.
g, G = slight, marked gas. }
* = colonies atypical.
† = negative on first isolation. ‡ = tested by von Wohlzogen Kühr's method.
nt = no test.
– = no reaction.
tr, ±, + = trace, moderate, marked reaction.
C = pale or dark cream. }
CL = café-au-lait. } Pigment on potato.
GB = grey-brown. }
SP = salmon pink. }

L318 markedly related to ER75 and slightly to 7810 and M90, and *Ps. icthyosmia* markedly to DF1*a* and slightly to 7812. M90 and ER75 formed unstable suspensions in 0·85 % saline, and both strains grew in two forms, a transparent colony whose constituent cells were long and filamentous, forming suspensions that were precipitated by 0·85 % saline on heating at 100° for 30 min.; and a more opaque colony consisting of saline- and heat-stable short rods; by virulence tests in frogs and mice the 'opaque' form of M90 was slightly more virulent than the 'transparent'; the two forms of ER75 were equally virulent.

Table 2. *Cross-agglutination of twelve strains examined by* Proteus hydrophilus *and* Pr. melanovogenes *antisera, and of* Pr. hydrophilus *and* Pr. melanovogenes *strains by* Aeromonas liquefaciens *and* Pseudomonas icthyosmia *antisera.*

		Titre with antisera to						
Bacillus	Strain	N.C.T.C. 7810	N.C.T.C. 7812	ER75	M90	L417	L418	N.C.T.C 8049
Pr. hydrophilus	N.C.T.C. 7810	**80**	10	0	10	80	80	10
	N.C.T.C. 7812	10	**320**	0	160	20	20	20
Pr. melanovogenes	ER75	0	0	**160**	0	0	>160	0
	M90	40	320	10	**640**	10	10	10
'Red-leg'	DF1*a*	0	0	0	10	0	0	>160
	DF1*b*	10	0	10	10	10	10	0
	DF2	50	50	0	50	20	10	0
	DF3	0	20	0	0	>160	0	0
Aeromonas liquefaciens	L417	160	20	40	40	**320**	0	40
	L418	10	0	80	10	10	**>320**	0
Aeromonas hydrophilus	L447	40	>160	0	80	0	10	10
Ps. icthyosmia	N.C.T.C. 8049	0	20	0	0	0	0	**80**

0 = titres < 10. Homologous titres in heavy type.

It is possible that we were dealing with S and R states of M90, but a detailed analysis was not possible, because R → S and S → R occurred readily in overnight culture. The nature of the two stages in ER75 is less clear. Both N.C.T.C. 7812 and N.C.T.C. 7810 were consistently in the Smooth state.

Pathogenicity. With exceptions already noted, fatal bacteraemia within 36 hr. of intraperitoneal injection in mice. In frogs, with exceptions already noted, fatal red-leg with bacteraemia, many animals dying in tetanic spasm. (N.C.T.C. 7810 produced no symptoms of red-leg, perhaps because of its high virulence; the frogs died with a copious bacteraemia in < 18 hr.)

Saprogenicity. Characteristic black-rot produced in hen eggs.

DIFFERENCES OF THE TWELVE STRAINS STUDIED

Table 1 summarizes the outstanding differences in the twelve strains. By itself the lophotrichy of ER75 and DF3 does not necessarily separate them from the rest, because within well-recognized 'monotrichate' species, moderate lophotrichy is not uncommon (cf. Bartholomew, 1949). None of the strains differs consistently from the others in respect even of the majority characters listed.

Three of the DF strains ferment lactose with moderate vigour. Most strains produced acid after 1 day's growth in glucose phosphate medium, but this was neutralized by basic substances produced during the 2–7 days required in the standard M.R. test (see 'Committee', 1947). DF1*b* did not reverse the acidity.

In 1937, Miles & Halnan's strains of *Proteus melanovogenes* readily fermented arabinose. ER75 and M90 no longer do so, but in all other respects they behaved in our hands as described by Miles & Halnan. During the years of maintenance in soft nutrient agar they had lost some of the power to produce rot and some of their virulence for mice, but the virulence was restored by selection of colonies in the Smooth state and by frog and mouse passage. Their saprogenicity was not improved either by egg or mouse passage.

With regard to the authentic *Pr. melanovogenes* and *Pr. hydrophilus* strains, there is, compared with the large numbers of common characters listed in the preceding section, nothing in the differences to justify maintaining the separations of the two species; ER75 and L418 are the most divergent, and are serologically closely related to one another and alone of the group fail to ferment dextrin and glycogen.

Where the results of Koser's citrate test and of cultivating in citrate-$(NH_4)_2SO_4$ differed with each strain, the differences were consistently obtained in several repetitions of the tests; due precautions were taken against the carry-over of carbon sources other than citrate when seeding the media. These differences may reflect real differences in biochemical constitution, but as far as the bare fact of citrate-utilization is concerned, the twelve strains are all positive.

If we allow that the divergence displayed by the first four strains listed in Table 1 is insufficient to maintain *hydrophilus* and *melanovogenes* as separate species, and agree to class them as *hydrophilus*, there is little reason for excluding the DF strains from the species, though the ready fermentation of lactose perhaps denotes a noteworthy variety; but even this character was lost by DF3 after a few subcultures. The fact that all the DF strains induced red-leg in frogs and rot in eggs is an added reason for their inclusion with the rest. The fact that they were isolated from red-leg in frogs is also suggestive, but is not in our view decisive, because we deprecate the use of habitat as a taxonomic character of microbes. The limitations of habitat, including hosts that are parasitized, as a distinctive character in bacterial classification, are well illustrated in *Pr. hydrophilus*, which, if *defined* as the cause of red-leg, must be restricted to the microbe found in diseased reptiles and amphibians—a useless restriction when the ecological significance of bacilli of this kind is under consideration. Moreover, haemorrhagic infections in these animals may be caused by microbes other than *Pr. hydrophilus*. Thus a typical outbreak of red-leg in green tree-frogs was produced by *Bacterium alkaligenes* (Miles, 1950).

Bergey's Manual (1948) records the liquefaction of gelatin by *Proteus hydrophilus* as 'napiform', the utilization of citrate as positive and the fermentation of lactose as negative. We are not inclined to regard napiform liquefaction as definitive. In our hands, the liquefaction varied between saccate, infundibuliform and napiform, and one strain could be made to produce liquefied masses of different shapes in stab cultures by varying the concentration of gelatin and the amount of bacterial growth seeded. All our strains except N.C.T.C. 8049 and L418 grew in a citrate medium with $(NH_4)_2SO_4$ as a source of N, and were readily subcultured in the same medium; but we were unable to subcultivate all the strains serially in Koser's citrate, although N.C.T.C. 8049 and L418 grew quite readily in this citrate medium. With regard to lactose, the slight acid produced by most strains in 4 days at 37° in 1 % lactose in peptone water is not frank fermentation; but five strains produced marked acidity, three of them in 1 day at 37°, and it would be as well, pending future work on newly isolated strains, to characterize the action of *Pr. hydrophilus* on lactose as variable, but usually feeble.

THE CLASSIFICATION OF *PROTEUS HYDROPHILUS*

There is no reason to doubt that *Pr. hydrophilus* is the bacillus described by Sanarelli (1891) as *Bacillus hydrophilus fuscus*, and that, if Sanarelli's description is the first, with due avoidance of a trinomial, *B. hydrophilus* Chester (1901) gives the valid specific epithet. One species that resembles *Proteus hydrophilus* was described in 1890 by Zimmerman—*Bacillus punctatus*. Zimmerman's description, however, is very scanty. *B. punctatus* corresponds to *Proteus hydrophilus* in size, rapid motility by one polar flagellum, turbid growth in broth with pellicle, brownish pigment on potato, and saccate liquefaction of gelatin. Its eponymous character, the production of white punctate colonies floating on the surface of liquefied gelatin, may be accidental. None of our twelve strains had it, when tested on 15 and 20 % nutrient gelatin plates. For these reasons we think that *Bacillus punctatus* has no good claim as a recognizable first description of a species, and that the specific epithet *hydrophilus* should be regarded as established for this species.

The generic name, on the other hand, is more doubtful. If with Bergey (1948) we limit the genus *Proteus* to peritrichate bacilli that split urea, clearly *hydrophilus* belongs elsewhere. Its monotrichy suggested the genus *Pseudomonas* to Guthrie & Hitchner (1943), but Stanier (1943) objected on the grounds that the 'true fermentative ability (*sensu* Pasteur)' of *Proteus hydrophilus* was not characteristic of typical *Pseudomonas* spp. With this objection we agree; some other genus is required. Stanier observed in *Pr. hydrophilus* the 2:3-butanediol fermentation described by Beijerinck (1900) for his *Aerobacter liquefaciens*, a Gram-negative bacillus isolated from the dried rhizome of *Althaea officinalis*. Kluyver & van Niel (1936) proposed the genus *Aeromonas* for bacilli in the family Pseudomonadaceae that ferment glucose with the production of 2:3-butanediol. According to Stanier, Beijerinck's microbe should therefore be *A. liquefaciens*. It resembles *Pr. hydrophilus* closely, except that its growth was optimum about 28° and often inhibited at 37°. If we regard optimum growth temperature as an accidental and not specific character, then 'liquefaciens' must give way to the earlier epithet 'hydrophilus'. We also concur with Stanier's view that *Pseudomonas fermentans* von Wohlzogen Kühr (1932) is *Proteus hydrophilus*. We repeated all the tests that von Wohlzogen Kühr describes in his paper, and in all of them our twelve strains, which include two authentic strains of *Pr. hydrophilus* and an authentic strain of *Pseudomonas fermentans*, behaved alike. The pigment production by *Ps. fermentans* does not appear to have been sufficiently marked to warrant the description 'chromogenic' (Bergey, 1934), though six out of our twelve strains produced a definite salmon pink colour on potato which, however, varied in degree with repetition of the potato culture. The other six strains grew with the production of pigment varying from creamy white to pale café-au-lait (Table 1).

CONCLUSIONS

We suggest that: (*a*) *Bacillus punctatus* Zimmerman be recognized as insufficiently described, and therefore without status as regards priority in naming; (*b*) *Aerobacter liquefaciens* Beijerinck may be synonymous with *Bacillus hydrophilus fuscus* Sanarelli and *Proteus hydrophilus* Bergey *et al.*, and certainly belongs to the same genus as these two species; (*c*) *Pseudomonas fermentans* von Wohlzogen Kühr, *Proteus melanovogenes* Miles & Halnan and *Pseudomonas icthyosmia* Hammer are synonymous with *Proteus hydrophilus*; (*d*) if the production of 2:3-butanediol in the fermentation of glucose proves to be a property characterizing an otherwise substantially homogeneous group within the Pseudomonadaceae, the genus *Aeromonas* Kluyver & van Niel should be accepted.

The recognition of the type species and the authenticating of type-cultures must await the establishment of *Aerobacter liquefaciens* and *Proteus hydrophilus* as two species or one. If two, the type species is clearly *Aeromonas liquefaciens*; if one, we suggest that the type species is *A. hydrophila*, and the type culture should be an authentic strain of *Proteus hydrophilus* Bergey *et al.*

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The Mouse Pathogenicity and Toxicity of *Proteus vulgaris*

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SUMMARY: The LD50 for mice of twenty-one strains of *Proteus vulgaris* from human infection ranged from 2 to 600 million living bacilli. Mouse-virulence was not associated with the severity or the site of infection in the human subject.

The relatively high virulence of some strains was not due to any peculiar demonstrable toxicity of the cell-substance, because killed cells of high and low virulent strains were about equally toxic. The toxicity appeared to reside in a predominantly lipo-polysaccharide fraction, immunologically similar to the antigen characterizing the somatic surface of recently isolated strains.

This investigation was made to find whether strains of *Proteus vulgaris* isolated from infections in man could be distinguished by a laboratory virulence test, and whether virulence could be correlated with any other microbiological character of the strains.

MATERIALS AND METHODS

For the purposes of this paper the name *Pr. vulgaris* is used for all urease-positive, swarming strains of the Proteus group, withholding the distinction of *Pr. vulgaris* and *Pr. mirabilis* until the specific status of these two groups is more firmly established (Topley & Wilson, 1946, p. 649).

Source and properties of the strains. Twenty-one strains of *Pr. vulgaris* were isolated either from the urine of patients with pyelitis or cystitis or from the pus found in various situations—the mastoid antrum, lung and pleural abscesses, and acutely infected surgical wounds. No strain was included in the survey unless it was either the sole or the predominant organism on blood and MacConkey agar plates seeded directly with the pus or a spun deposit of the urine; that is, unless it gave at least this indication of being the chief cause of the infective lesion from which it was isolated. The strains were maintained in stab-cultures in nutrient broth + 0·75 % agar, in which they could be stored for several months in the cold (2°–8°) without subculture.

All strains swarmed on nutrient 2 % agar media. They fermented glucose rapidly with production of acid and gas, but did not ferment mannitol, lactose, arabinose, dulcitol, inositol, rhamnose. All but PR8 and PR24 (see below) slowly produced acid from sucrose and salicin: and all but PR14 fermented xylose in 2–5 days with the production of acid and gas; PR14 produced slight acid from xylose in 10 days.

All strains produced slight acid, then marked alkali and peptonization in litmus milk, liquefied gelatin rapidly, digested Loeffler's coagulated serum medium, and were catalase, nitratase, urease and H_2S positive. The utilization of citrate in Koser's medium was variable. Except PR5, none fermented maltose or produced indole and all produced acetylmethylcarbinol tested by

Barritt's (1936) method; PR 5 fermented maltose, was indole-positive and Voges-Proskauer negative.

PR 5 falls therefore into the group '*Pr. vulgaris* Hauser' and the rest into the group '*Pr. mirabilis* Hauser'.

Virulence tests; calculation of the LD 50. Within 1–2 weeks of isolating the strain, overnight subcultures from the stock stab cultures, in nutrient broth at 37°, were tested in 18–22 g. mice by intraperitoneal injection of 0·25 ml. graded ten-fold dilutions. Four or six mice per dose were used. At the same time a 6-drop viable count was made on MacConkey agar plates by the method of Miles & Misra (1938). Only those deaths accompanied by a bacteraemia with *Pr. vulgaris* cultivable from the heart blood on direct plating were recorded. All such deaths occurred within 7 days of injection. The virulence was measured by the LD 50, estimated by Gaddum's (1933) method. The method was not strictly applicable to some of the results obtained. Thus in six of the titrations the tenfold grading of doses was too coarse, one dose killing no mice, and ten times the dose, all the mice (e.g. Table 1*a*, PR 9). In these cases the LD 50 was estimated on the assumption that the highest non-killing dose was the maximum LD 0, that the lowest dose killing all the mice was the minimum LD 100, and that the intermediate LD values were distributed normally between these limits. In two titrations, PR 23 (Table 1*a*) and PR 24, the highest dose killed no mice; here the LD 50 is recorded as greater than the highest dose given, and (in brackets) as an LD 50 estimated on the assumption that this dose was the maximum LD 0 and that a dose 10 times as great would have killed all the mice, and was the minimum LD 100. In all these cases, therefore, the LD 50, recorded in Table 1*b* in ordinary type, can be regarded only as a very approximate measure of virulence; no estimation of the error is justifiable. For the remaining fifteen titrations, the responses were like those to PR 1 (Table 1*a*) and both LD 50 and the approximate error are recorded in Table 1*b* in heavy type.

Toxicity tests. The toxicity of whole killed bacilli, and of fractions derived therefrom, was estimated by intraperitoneal injection of graded doses in 18–22 g. mice, and Gaddum's method for calculating the LD 50 applied.

Precipitin tests were made by adding 0·3 ml. of a given concentration of rabbit antiserum against somatic antigens to 0·3 ml. of three-fold dilutions of antigen, and reading the end-point of just visible precipitation after 3 hr. at 50° and overnight on the bench.

RESULTS

Virulence of recently isolated strains

Table 1*b* records the source and the approximate LD 50 of the twenty-one strains. Though estimates are crude it is clear that virulence varies widely; and from column 2, that neither urinary nor pus strains were peculiarly virulent. The different estimations were made upon the laboratory strain of mice over a period of 10 weeks, and may be considered reasonably comparable, though not as comparable as would be the case had all strains been tested simultaneously on a single large batch of mice. (The strains were numbered in order of virulence estimated by inspection of the death rates. PR 3 and PR 17 particularly are out of place because in each case a single death with a few bacilli brought down the estimate of the LD 50.)

When the strains were isolated, notes were taken of the clinical severity of the infection; the mouse-virulence of the strains was not in any way associated with clinical severity. The LD 50 varies from 2 to 600 million living bacteria. Though the dose-response was steep in most cases, it was less steep for one of the most virulent strains, PR 1 (Tables 1*a* and 2); suggesting that whereas the death was largely toxic in most tests, PR 1 had an invasive capacity not displayed by the other strains.

Table 1*a*. *Examples of coarse titrations of virulence of Proteus strains*

Strain (PR)	Dose of viable bacilli (millions)	No. of mice dying/group
1	75·00	6/6
	7·50	4/6
	0·75	1/6
9	47·00	6/6
	4·70	0/6
	0·47	0/6
23	125·00	0/4
	12·50	0/4
	1·25	0/4

Table 1*b*. *The LD* 50 *in mice of twenty-one urinary and pus strains of* Proteus *spp.*

Strain	Source	LD 50 and approx. error (millions)
1	Urine	2·28 ± 0·87
3	Urine	0·23 ± 0·00128
4	Urine	10·6 ± 0·00081
5	Pus	11·0
7	Pus	13·0
8	Urine	11·7 ± 0·0057
9	Pus	44·0
10	Pus	15·0
11	Urine	15·8 ± 0·0077
12	Urine	17·0 ± 0·0083
13	Pus	22·0 ± 0·001
15	Urine	20·8 ± 0·0485
16	Urine	24·6
17	Urine	0·37 ± 0·0457
18	Urine	52·4 ± 0·00105
19	Pus	63·0 ± 0·007
20	Urine	93·0
21	Pus	55·0 ± 0·00153
22	Urine	160·0
23	Urine	> 56 (160·0)
24	Pus	> 125 (625·0)

Comparison of strains of presumed high and low virulence

The relation between the capacity to invade and to multiply in the body, and the gross toxicity of the bacilli, was explored in greater detail in a comparison of the two most virulent and the two least virulent strains—PR 1, PR 3, PR 23 and PR 24.

Eighteen-hour cultures from the heart blood of mice dead from infection by the strains were seeded on to nutrient agar in Roux bottles, and the cells harvested after 18 hr. growth at 37°. They were washed twice by centrifugation in sterile Ringer's solution and half of each suspension heated for 90 min. at 60°. These heated suspensions, which were subsequently proved to have been sterile, and the corresponding living suspensions were diluted five-fold, and viable counts made of the living suspensions. Samples of each killed suspension were dried over P_2O_5 *in vacuo*, and after due allowance for the contained salts of the Ringer solution, the weight of bacteria/ml. determined. The death-rates for fivefold differences in dose are recorded in Table 2.

Table 2. *The LD50 of living (L) and dead (D) Proteus strains, two (PR1 and PR3) of high, and two (PR23 and 24) of low mouse-virulence.*

PR1			PR3			
No. bacilli (millions)	No. mice dying/group		No. bacilli (millions)	No. mice dying/group L	No. bacilli (millions)	No. mice dying/group D
	L	D				
19,840	—	6/6	8675	6/6	86,750	6/6
3,968	—	2/6	1737	6/6	17,370	3/6
793	6/6	0/6	348	6/6	3,474	1/6
159	6/6	0/6	70	1/6	695	0/6
32	4/6	0/6	14	0/6	139	0/6
6	2/6	—	—	—	—	—
1	1/6	—	—	—	—	—
LD50 (millions) ...	10·8	6925 (≡0·954 mg.)		139		15,300 (≡1·23 mg.)

PR24			PR23			
No. bacilli (millions)	No. mice dying/group		No. bacilli (millions)	No. mice dying/group L	No. bacilli (millions)	No. mice dying/group D
	L	D				
17,550	—	6/6	3550	6/6	35,500	6/6
3,510	—	0/6	710	5/6	7,100	1/6
702	6/6	0/6	142	2/6	1,420	0/6
140	3/6	0/6	28	0/6	280	0/6
28	0/6	—	6	0/6	57	0/6
6	0/6	—	—	—	—	—
1	0/6	—	—	—	—	—
LD50 (millions) ...	140	12,800 (≡2·73 mg.)		244		11,350 (≡2·91 mg.)

These more accurate titrations confirm the relatively high virulence of PR1, but class PR3 with the low-virulent PR23 and PR24. The change in PR3 is great enough to suggest a loss of virulence even in the little manipulation and subculture that took place between the first and second tests. The dead bacilli, from the same cultures as the living, were more toxic in PR1, but the twofold difference is slight compared with the difference in virulence. The LD50 ratios for dead and living bacilli were: PR1, 595; PR3, 110; PR23, 48; PR24, 91.

The high ratio (595) for PR1 suggests that this strain is better than the others in multiplying in the body to the point of lethal intoxication, that is, it is more invasive. It is clear, therefore, in these strains, that toxicity *per se* is unlikely to be even a partial index of virulence. In any event, the large errors in the titrations suggest that prohibitively large numbers of mice would be required to establish significant differences in virulence.

The lethal substance in dead Proteus cells

Extraction of endotoxins. The four strains, PR1, PR3, PR23 and PR24, were tested as soon as possible after isolation, maintained with the minimum of subcultivation, and passed through mice for retest. If the qualities of *Pr. vulgaris* associated with virulence are similar to those of the S form of other Gram-negative bacilli of the Bacterium, Shigella and Salmonella groups, these procedures should have preserved the strains in their original virulent state. With variation to an avirulent state associated with loss of characteristic and perhaps toxic antigens, it was possible that toxicity might change greatly during artificial subculture.

Large quantities of bacteria were therefore harvested as soon as possible after the virulence tests, using as seed the primary stock stab cultures made from the heart blood of mice dying of Proteus infection. The toxicity of cultures killed by heating for 90 min. at 60°, and by precipitation from the culture medium by acetone, proved to be similar. Two to three-days' growth from nutrient agar in Roux bottles at 37° was accordingly washed off with 0·85 % saline, and precipitated with 3 vol. acetone, resuspended for 2 days in pure acetone, spun and suspended for 8 hr. in anhydrous ethyl ether and the spun deposit from the ether dried rapidly *in vacuo*. The rapid evacuation of the desiccator produced a crust of dried bacilli on the ether-wet deposit, and a few minutes later the superficially dry mass exploded to produce a fine, readily manipulable bacterial powder that could be stored indefinitely.

The toxicity of suspensions of virulent PR1, killed by heating to 55°, by ethanol, by 0·1 % formalin and by weak hydrochloric acid, were compared; they were equally toxic, weight for weight, as judged by a coarse titration. That is, there was no evidence of a grossly labile toxin destroyed at 60° or by acetone.

Trypsin and toxicity of Proteus cells. By analogy with *Salmonella typhimurium* (Raistrick & Topley, 1934) the predominant toxic substance in *Proteus vulgaris* should resist tryptic digestion and be liberated from the cells by it. The acetone-killing of cultures was adopted with this end in view. The limited acetone treatment in preparing the stock bacterial powder rendered the cells moderately susceptible to trypsin, but for complete and a more rapid digestion, continuous extraction of the bacterial powder with hot acetone (cf. Douglas & Fleming, 1921) for 24 hr. in a reflux condenser, which removed a trace of yellow oily material, was necessary. Suspensions of acetone-killed cells of each of the four strains, 2 mg./ml. of distilled water, were treated under toluene with a preparation of commercial trypsin (British Drug Houses Ltd.) 0·1 mg. to each ml. of suspension for 2 days at 37°, the pH being maintained at 8·3–8·5 by twice daily additions of NaOH. An equal quantity of trypsin was then added. After 3 more days at 37° and pH 8·3, digestion was almost complete, with a translucent yellowish supernatant fluid and a little amorphous cell debris. Suspensions without trypsin were maintained under toluene for 5 days at 37° at pH 8·3. For each strain, the volumes of digested and undigested suspension were made equal, and the LD 50 of the digests determined in relation to that of the starting material. The figures for PR 3 (Table 3) exemplify the four results, which were:

Strain PR	Ratio of LD 50 digest/cells
1	0·54
3	1·31
23	0·96
24	1·03

Table 3. *The LD 50 of acetone-killed suspensions of PR 3, before and after tryptic digestion*

Dose (mg. of original dry bacilli)	No. mice dying/group injected with	
	Untreated bacilli	Digested bacilli
15·00	6/6	6/6
5·00	6/6	4/6
1·66	1/6	2/6
0·55	0/6	2/6
LD 50 ...	2·49 mg.	1·90 mg.

The limits of error of the ratios were large, and there is no significant alteration in toxicity by digestion, though the twofold decrease in toxicity of PR 1 is suggestive. It is possible that in any one digestion, digestion might destroy one toxin and liberate an equitoxic amount of another, but such a coincidence is unlikely to occur in three instances, and it appears that the endotoxin of PR 3, PR 23 and PR 24, like that of *Salmonella typhi-murium*, is resistant to tryptic digestion. On the other hand, trypsin may have destroyed some toxin in PR 1.

Centrifugation of the digests deposited considerable amounts of material. The distribution of toxicity between the deposit and the remaining soluble substances was measured in PR 1 and PR 23. For each strain, a batch of dried cells was suspended in water, half the suspension being incubated under toluene at pH 8·5, and half digested with trypsin under the same conditions. The solids in the digest were spun out at 3000 r.p.m., leaving a turbid fluid from which a translucent gel was deposited at 18,000 r.p.m., or at 3000 r.p.m. after treatment of the fluid with 10 % by weight of ethanol. This gel was amorphous under ×1000 magnification, but on suspension in water gave a slightly turbid, shimmering suspension that precipitated specifically with O-antisera. For the toxicity test, all the solids precipitated by treatment of the digest with 0·14 vol. ethanol and washed with water containing 10 % (w/w) ethanol, were used (insoluble). The weight of material in 'insoluble' and soluble fractions was determined; and the recovery of toxic substances estimated (Table 4). Most of the toxin was in the soluble fraction, though tryptic digestion and perhaps the mild ethanol treatment had in each case destroyed about one-third of the toxicity.

Table 4. *The toxicity of tryptic digest of Proteus bacilli soluble (S) and insoluble (I), and untreated bacilli*

Strain	Material	LD 50 (mg.)	Total weight material (mg.)	Recovery of toxic substances (%)	
PR 1	Untreated	1·68	612	(100)	
	Soluble	2·80	620	64·0	67·5
	Insoluble	7·01	90	3·5	
PR 23	Untreated	3·00	601	(100)	
	Soluble	4·63	616	53·3	61·3
	Insoluble	4·07	80	8·0	

Ethanol fractionation of tryptic digests. Raistrick & Topley (1934) precipitated the *typhi-murium* endotoxin from their tryptic digests by 68 % by weight of ethanol. Dried cells (3 g.) of each of the four Proteus strains in 600 ml. water were digested by two 75 mg. lots of trypsin. After 5 days at 37° the digests were concentrated at 45° to 120 ml. and solid matter ('insoluble' fraction FI) spun off at 18,000 r.p.m. The solids were washed and the washings added to the fluid, which was then concentrated to 40 ml., brought to pH 7·0, and mixed with 68 % by weight of ethanol. The precipitate (F 68) was removed, and the weight of added ethanol brought to 90 %. After removal of the precipitate (F 90) the liquor was finally treated with an excess of acetone and ethyl ether (80 % + 20 %, v/v), to obtain the FAE fraction. All the precipitates were washed with acetone and dried *in vacuo* over H_2SO_4 and NaOH pellets. FI and F 68 were fine white powders, but both F 90 and FAE were yellowish, pasty and deliquescent. From the 3·3 g. of starting material (cells + trypsin) 85–95 % of solids were recovered (Table 5), the yields of F 68 lying between 24·8 and 30·1 %.

Table 5. *Percentage yields from fractionation with* 68 % (*w/w*) *and* 90 % *ethanol, and an acetone-ether mixture*

Fraction strain ...	PR 1	PR 3	PR 23	PR 24
	Yields (%)			
F1	6·4	8·2	8·3	10·4
F 68	24·8	27·4	28·6	30·1
F 90 + FAE	54·0	56·2	55·1	54·5
Recovery of solids	85·2	91·8	92·0	95·5

Properties of the F 68 fraction. Though, especially in PR 1, up to half the toxicity in dried cells of *Proteus vulgaris* is destroyed or lost during digestion and ethanol fractionation, the F 68 fraction nevertheless appears to be a representative though an impure sample of the endotoxin of *Pr. vulgaris*. First, the yield was similar from all recently isolated strains; from the twenty-one strains thus fractionated, the yield lay between 24·1 and 35·7 %, with an average of 30·2 %. Secondly, the LD 50 of F 68 prepared in parallel from PR 1, PR 3, PR 23 and PR 24 were respectively 1·94, 1·63, 2·41 and 3·12 mg. which are roughly of the same relative magnitudes as the toxicities of the heat-killed cells reckoned as dry weight of the cells (0·95, 1·23, 2·73 and 2·91 mg., Table 2). Thirdly, their toxicity and precipitability by specific O-antisera were, in comparison with other fractions, roughly parallel.

Finally, the immunological specificity of the F68 fractions was paralleled by that of the living bacilli, as regards serological reactions and immunizing power. The O antigen of PR1 was distinct from those of PR3, PR23 and PR24; PR23 and PR24 were identical, but PR3, though closely related, was not identical with them (Belyavin, Miles & Miles, 1951). When cross-testing the survivors of many virulence and toxicity tests of these strains with homologous or heterologous living bacilli, it was observed that the whole cells of PR3, PR23 and PR24 were equally good in immunizing against infection by any one of the strains, whereas there was little cross-immunity between PR1 and the other three.

The F68 fractions behaved similarly. In one test, the F68 of PR1 in intraperitoneal doses of as little as 1 µg. conferred a solid immunity against infection by two LD100 of living PR1 given 1 week later (Table 6*a*). Similar doses were also effective, though less so in another test (Table 6*b*) against *c.* LD90 of PR1, and ineffective against *c.* LD90 of PR3, both given 18 days after immunization. The specificity was, however, comparative, not absolute; large doses of F68 from PR1 gave some protection against PR3 infection and the F68 of all four strains could elicit hypersensitive (allergic) reactions in the skin of rabbits hyperimmunized with any one of them.

Table 6. *Immunizing power of the F68 fraction of strain PR1*

		Deaths after infection by			
		PR1		PR3	
	Immunizing dose (µg.)	Millions of organisms	No. mice dying/group	Millions of organisms	No. mice dying/group
(*a*)	25	305	0/10	—	—
	5	305	0/10	—	—
	1	305	0/10	—	—
	0	305	10/10	—	—
	0	61	2/10	—	—
	0	12	1/10	—	—
(*b*)	50·0	800	1/10	775	10/10
	5·0	800	4/10	775	9/10
	0·5	800	4/10	775	9/10
	0·0	800	9/10	775	9/10

DISCUSSION

There have been a number of attempts to measure the mouse-virulence of *Pr. vulgaris* (e.g. Yacob, 1932) of human origin, but the results are mainly crude, like the observation that whereas 1 ml. of an overnight culture was fatal, 0·2 ml. was not. My titrations are less crude, though large numbers of mice would be needed to distinguish even tenfold differences in LD50 in a single comparison; and unexplained variability in Proteus strains makes it likely that an LD50 so obtained would not be consistently observed with repetition of the test. Nevertheless, it is clear in general that the mouse-virulence of *Pr. vulgaris* is low, but that some strains, soon after isolation at least, have a low LD50 that suggests invasive powers above the average.

Most of the tests were made in 1937, as part of an investigation interrupted by the 1939–45 war, and cultures were not preserved in the dry state soon after isolation. With the better current methods of storing cultures, it might be possible to determine the factors responsible for the greater invasiveness of some strains. The relatively brutal analysis to which the strains were subjected—heat- or acetone-killing, digestion, and fractionation with organic solvents—is likely to have revealed little but stable components of the bacillary cells that might be associated with virulence; but there was no indication of a toxin associated with the more virulent strains.

Previous studies of the toxins of *Pr. vulgaris* have been mainly of culture filtrates, though Triffterer (1943) records that trichloroacetic acid extracts of Proteus X19 were toxic for mice, and that the toxicity was neutralized by the sera of typhus patients.

My fractionation of endotoxic substances in the Proteus cells is only preliminary, and much remains to be investigated. The F68 fractions, which by crude tests appeared to contain a large proportion of lipo-polysaccharide, are probably similar to the alkali-labile O-receptor of White (1933), the P-substance of Castaneda (1935), the glyco-lipid antigen of Ciuca, Mesrobeanu, Badenski & Muteanu (1938), all of which were obtained from Proteus X19; and to the T-1 fraction that Bendich & Chargaff (1946) obtained after removing from tryptic digests all material sedimentable by high-speed centrifugation. All these fractions appeared to be characteristic of Proteus, as distinct from rickettsial cells, and it is noteworthy that limited tests suggest that most of the toxicity of dead cells remains in this F68 fraction (Table 4); much less is present in the 'insoluble' fractions corresponding to Bendich and Chargaff's T-2, the immunologically distinct antigen that characterizes both Proteus and rickettsial cells.

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Isolation of a Bacterium, Producing Propionic Acid, from the Rumen of Sheep

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SUMMARY: A strictly anaerobic micrococcus which produces acetic acid, propionic acid, carbon dioxide and hydrogen by fermentation of lactate was isolated from the rumen of sheep. It failed to ferment sugars and was identified as *Veillonella gazogenes*. Its significance in ruminant digestion is uncertain, but a characteristic reaction of this organism, the decarboxylation of succinic acid to yield propionic acid and carbon dioxide, appears to be important in the rumen. This reaction was studied by the Warburg technique with washed suspensions of rumen bacteria.

It had been known for a considerable time that volatile acids were amongst the products of rumen fermentation of plant material, but it was not until Elsden (1946) developed a partition chromatographic method which separated quantitatively mixtures of fatty acids that their identity was definitely established. Acetic, propionic and butyric acids were always found and traces of higher acids were sometimes detected. Very little is known about the micro-organisms responsible for the digestion of carbohydrate in the ruminant or of the metabolism of the organisms active in rumen fermentation. It is now generally agreed that the main role in rumen fermentation is played by bacteria, since the removal of protozoa by drenching with dilute copper sulphate has little effect on the digestive process (van der Wath, 1942).

Most of the attempts to isolate organisms responsible for the chemical changes in the rumen were made on digesters of cellulose. The most successful approach to this difficult problem was made by Hungate (1946, 1947). In the present work an attempt was made to isolate organisms responsible for the production of propionic acid in the rumen of sheep. Elsden (1945) isolated, from cellulose-enrichment cultures of rumen contents, bacteria which appeared to be members of the genus *Propionibacterium* and which he believed were responsible for the formation of the propionic acid invariably found in the rumen. Elsden stated that: 'Under the conditions used, the fermentation of cellulose appears to be a two-stage process, a primary breakdown of cellulose by the specific organisms followed by a secondary fermentation of the product or products thus formed by the propionic acid bacteria.' Since all propionic acid organisms so far isolated ferment lactic acid, with the exception of *Propionibacterium acnes* (Douglas & Gunter, 1946), and since lactic acid appears under certain circumstances (Phillipson, 1942) to be an intermediate in the fermentation of cellulose to volatile fatty acids in the rumen, it seemed logical to use enrichment cultures with sodium lactate as source of carbon for isolating the organisms responsible for propionic acid production in the rumen. Further, since the conditions in the rumen favour the growth of anaerobic bacteria, the enrichment cultures were incubated anerobically.

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EXPERIMENTAL

Methods

Isolation of bacteria. Glass-stoppered bottles of about 80 ml. capacity were filled with medium which was composed of the general purpose salt mixture of Stephenson (1949), with 0·4 % (w/v) Difco yeast extract and 1 % (v/v) sodium lactate, adjusted to pH 7. For the first enrichment 5 ml. of rumen contents, obtained from sheep with a permanent rumen fistula, were added to the bottles and the stoppers inserted so that no air bubbles were trapped. The bottles were incubated at 37° for 24 hr., by which time turbidity was evident and considerable evolution of gas had occurred. Two transfers were made at 24 hr. intervals using the same medium. Bacteria were isolated by plating on enrichment media solidified with 2 % (w/v) agar. The plates were incubated in a McIntosh & Fildes jar in an atmosphere of 95 % H_2+5 % CO_2. Growth of colonies was definitely better in this mixture than in pure hydrogen.

Determination of number of bacteria. The number of lactate-fermenting anaerobic bacteria present in rumen contents was determined by a serial dilution method using two series of tubes, one series (*a*) containing enrichment medium and the other (*b*) enrichment medium without lactate. Nineteen ml. of the medium + 2 % (w/v) agar + 0·02 % (w/v) $Na_2S.9H_2O$ were placed in test-tubes. The tubes were kept at a temperature of 50°, rumen contents (1·0 ml.) added, the solution thoroughly mixed, a 1 ml. sample withdrawn under sterile conditions and added to a second tube containing 19 ml. of the medium and so on for ten dilutions. The tubes were incubated anaerobically at 37° for 48 hr. The number of colonies present in the lowest dilution showing growth was noted. The agar was expelled from the tubes and the colonies at the lowest dilution picked out, Gram-stained and examined microscopically.

Preparation of washed suspensions of rumen organisms. Rumen contents were strained through muslin and differentially centrifuged as follows. The centrifuge was allowed to reach 200 r.p.m., and then switched off. A large proportion of the plant material had then settled down. The supernatant was next centrifuged at 2000 r.p.m. for 5 min., removing the remaining plant material, protozoa and some large bacteria. The supernatant was then centrifuged for 30 min. at 2000 r.p.m. and the resulting bacterial deposit resuspended in distilled water containing 0·02 % (w/v) $Na_2S.9H_2O$. This was spun down yielding a bacterial deposit equivalent to *c.* 0·5 g. dry weight bacteria per 500 ml. rumen contents. These bacteria were taken up in distilled water containing 0·02 % (w/v) $Na_2S.9H_2O$.

Lactic acid was determined by the method of Friedemann & Graeser (1933).

Propionic and acetic acids were identified and estimated by the silica gel partition chromatographic method of Elsden (1946). Martin & Synge (1941) showed that R, the ratio between the rate of movement of a compound down the column and the rate of movement of the developing solvent in the tube, is related to the partition coefficient of the compound between the water and the non-polar phase. The value of R is thus characteristic of the compound. In the case of the volatile fatty acids the R values vary with the concentration, and with different batches of silica gel (Elsden, 1946). In identifying acids it was thus necessary to determine both the concentration and the corresponding R value for the particular preparation of silica gel. The individual acids after resolution on silica gel were chromatographed together with known acids as markers to verify the identifications.

Apparatus for fermentation-balance experiment. The fermentation was carried out in a flask attached to a mercury-filled manometer connected to an adjustable reservoir, which was adjusted to keep the gas at atmospheric pressure. It was so arranged that after inoculation under sterile conditions the apparatus could be filled with sterile nitrogen. The volume of gas produced by fermentation included that indicated by the mercury manometer and the dissolved gas in the liquid (mainly CO_2) which was determined in the van Slyke manometric gas-analysis apparatus. The gas in the manometer was analysed by means of the Haldane gas-analysis apparatus.

Characters of bacteria isolated

The colonies formed on the agar plates were all of the same kind, clear, convex, smooth, circular and entire, greyish white by reflected light and of characteristic honey colour by transmitted light. The cells were spherical, 0·3–0·6 μ. in diameter, occurring singly, in pairs and in large irregular groups; Gram-positive in young cultures but Gram-negative after 12–18 hr. growth. The organism did not liquefy gelatine, was non-haemolytic, H_2S positive, indole negative and reduced nitrate to nitrite. Most strains appeared to be weakly catalase positive but this varied from strain to strain. Strictly anaerobic conditions were essential for growth, with temperature and pH optima 37° and 6·0–8·0 respectively. The following compounds were fermented vigorously: DL-lactate, pyruvate, L-malate, fumarate and D-tartrate.

The following compounds were not fermented: glucose, fructose, arabinose, L-xylose, galactose, mannose, maltose, L-sorbose, sucrose, lactose, raffinose, rhamnose, inulin, starch, glycerol, dextrin, mannitol, salicin, citrate, erythritol, DL-serine, alanine, glutamic acid, glycine, methionine, L-cystine, tryptophan, sodium formate.

From these characters it seemed likely that these organisms were similar to the Gram-negative anaerobic micrococci isolated from saliva by Hall & Howitt (1925). Hall & Howitt considered these organisms to be identical with *Micrococcus alcalescens anaerobius* described by Lewkowicz (1901) and renamed it *M. gazogenes*. Bergey (1948) adopted the classification of Prévot (1933) who placed it in the genus *Veillonella* of the family Neisseriaceae.

Strains of *Veillonella gazogenes* (*alcalescens* var. Fay) obtained from Dr A. R. Prévot were identical in fermentation tests and staining reactions with the strains isolated in the present investigation. The products of lactate fermentation were also identical, i.e. lactate was fermented to H_2, CO_2, acetate and propionate. Foubert & Douglas (1948*a*) obtained from tonsils anaerobic micrococci which gave these fermentation products with lactate as substrate and which they considered to be identical with *V. gazogenes*. They observed that the organism was Gram-positive in young cultures, making it ineligible for classification under the family Neisseriaceae, and they suggested transferring it to the genus *Micrococcus*, giving it the species name *lactilyticus*.

It is believed that the micrococcus isolated from the rumen in the present work is *Veillonella gazogenes*. It was found in the rumen of all sheep examined and was isolated from sheep saliva and from the human mouth. Elsden & Volcani (private communication) isolated two strains with similar properties from the rumen of sheep in California.

Fermentation balance experiments

Table 1 gives the result of a typical balance experiment carried out with strains of *Veillonella* ((*a*) isolated from the rumen of a sheep; (*b*) Dr Prévot's organism) in the lactate medium used for isolating the bacteria.

Table 1. *Products of fermentation of lactate by* Veillonella *strains at* 37°

Concentrations of products are given in mmol./100 mmol. lactate fermented.

	Source of organism	
Products	Rumen	Institut Pasteur
Propionic acid	58·5	56·0
Acetic acid	48·7	45·0
Carbon dioxide	35·2	38·0
Hydrogen	14·8	22·4
Carbon recovery (%)	102·0	98·5
Redox index	1·04	1·07

The oxidation-reduction (redox) index was calculated by the method introduced by Johnson, Peterson & Fred (1931). It is important to calculate the redox balance as well as the carbon balance as it gives an indication whether a product has been overlooked; it may also give an indication of CO_2 fixation (Wood & Werkman, 1936).

The results in Table 1 are similar to those obtained by Foubert & Douglas (1948*b*) with the organism they designated *Micrococcus lactilyticus*. It will be shown in a subsequent paper that the ratio in which the products are obtained is not a constant, but is dependent on the CO_2 concentration in the medium.

Both the *Veillonella* strains isolated from the rumen and those obtained from Dr Prévot grew profusely on D-tartrate. A balance experiment was carried out using a *Veillonella* strain from the rumen and D-tartrate as the source of carbon (Table 2).

Table 2. *Fermentation of* D-*tartrate by a* Veillonella *strain from the rumen*

Concentrations of products are given in mmol./100 mmol. of D-tartrate fermented.

Propionic acid	8·8	Carbon dioxide	174·0
Acetic acid	95·4	Hydrogen	74·0
Carbon recovery (%)	97·7	Redox index	1·10

Importance of Veillonella *in the rumen*

The rumen population contains such a great variety of microbial species that mere isolation of an organism from rumen contents gives no information about its function in digestion. Elsden & Phillipson (1948) remarked that 'Two criteria must be fulfilled before an organism can be said to be a functional member of the rumen population: (*a*) the organism must perform a chemical reaction known to occur in the rumen; (*b*) the organism must be present in sufficient numbers to perform this reaction.' Criterion (*b*) might be modified to read: the organism must be present in sufficient numbers to perform this reaction extensively enough to account for a considerable part of the reaction rate observed in the rumen. With regard to criterion (*a*) it was shown that washed suspensions of *Veillonella* isolated from the rumen (Johns, 1948, 1949) characteristically and rapidly decarboxylated succinic acid and gave a quantitative yield of propionic acid and CO_2. The optimum pH for this reaction was between 5·9 and 6·0, with Q_{CO_2} 270–300. If *V. gazogenes* is present in large numbers in the rumen it would be expected that washed suspensions of rumen bacteria would also show decarboxylase activity with a similar pH optimum.

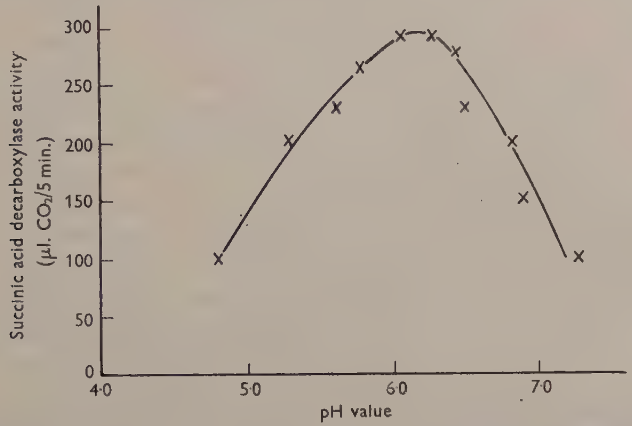

Fig. 1. Succinic acid decarboxylase activity of rumen bacteria at different pH values. Manometers contained: 1 ml. of bacterial suspension, 22 mg. dry weight/ml.; 1·6 ml. buffer 0·067 M phosphate, or phthalate below pH 5·3 (main compartment); 0·2 ml. 0·1 M succinate (side bulb 1); 0·2 ml. 2 N-H_2SO_4 (side bulb 2); gas phase N_2; temp. 37°. Two manometers at each pH; in one, acid tip at start, in other, acid tip after 5 min.

Rumen contents were obtained from sheep 16 hr. after having been taken off winter pasture (July) in New Zealand. The washed suspension obtained was tested for succinic decarboxylase activity by the Warburg technique. At pH 6·0 it was found that sodium succinate was quantitatively decarboxylated to propionic acid and CO_2. This experiment was repeated at different pH values (Fig. 1). From pH 5 to 7 there was considerable activity with a Q_{CO_2} of 134 at pH 6·2. Elsden (private communication) also found that washed suspensions of rumen bacteria decarboxylated succinate.

The number of *Veillonella* present was determined by serial dilution in duplicate using 1 ml. of rumen contents. The colonies in the lowest dilution were counted and then isolated after breaking the test-tube and expelling the agar into a sterile Petri dish. Determinations on three different animals which had been 16 hr. off winter grass gave: $7{\cdot}4 \times 10^6$, 1×10^6, $6{\cdot}2 \times 10^5$ organisms/ml. rumen contents. It is difficult to give comparative figures for other bacteria or even total numbers in the rumen as the methods employed by other workers have been different. Van der Wath (1942, 1948), by a counting technique which appeared to give reproducible results, found a total population on a diet of wheat straw of $5{\cdot}8 \times 10^8$ bacteria/ml.; when fish meal was added the population increased to $1{\cdot}9 \times 10^9$ cells/ml. Johnson *et al.* (1942) made total counts of a yeast-like organism which develops in the rumen of sheep when lucerne or mangolds are fed and found $2{\cdot}5$–$8{\cdot}3 \times 10^7$ organisms/ml. Gall, Burroughs, Gerlaugh & Edgington (1949*a*) found that bacterial slide counts from cattle and sheep on winter rations averaged about 5×10^{10}/g. rumen contents, while on pasture cattle and sheep averaged $9{\cdot}6 \times 10^{10}$/g. rumen contents and $8{\cdot}54 \times 10^{10}$/g. rumen contents respectively; Gall, Lorraine, Smith, Becker, Stark & Loosli (1949*b*), in a study of cobalt-deficient sheep, give bacterial slide counts in cobalt-fed sheep of $5{\cdot}46 \times 10^{10}$/g. rumen contents. The slide counts are total counts, of course, and include dead and viable bacteria. These figures appear to be too low for New Zealand conditions, as I have repeatedly found by the serial dilution method an unidentified organism which gives a count of the order of 10^{10}/ml. Until further work is done under New Zealand conditions the significance of the *Veillonella* counts and the degree to which criterion (*b*) is fulfilled cannot be assessed.

DISCUSSION

The biochemical changes which are brought about by the rumen microflora have been studied previously *in vivo* or by incubating rumen contents *in vitro*. Both methods are open to objections. When a particular substrate is added the natural population balance is upset by the multiplication of these organisms which readily utilize the particular substrate. *In vivo* sampling errors are a major difficulty, especially with cows, and an unknown amount of substrate end-products is continually being removed by absorption through the rumen wall and by passage down the alimentary canal. Pearson & Smith (1943), using the microscopical appearance of the bacterial flora and the pH of material incubated *in vitro* as criteria, believed that it was still comparable with normal rumen contents after 2–4 hr. incubation.

In the present work the washed suspension or 'resting bacteria' technique (Quastel & Whetham, 1925), which was developed for pure cultures of bacteria, was applied to the mixed culture obtained on centrifuging down rumen contents. This makes use of a rather arbitrary fraction of rumen bacteria according to the centrifuging procedure. Provided the experiment does not last longer than 2–3 hr. multiplication does not occur (Cook & Stephenson, 1928) as sources of nitrogen are removed by the washing. This technique should be useful for studying carbohydrate metabolism of rumen bacteria and though not yielding absolute figures, should, by the use of a standardized technique, give an idea of comparative biochemical activity.

V. gazogenes fulfils the first criterion of Elsden & Phillipson (1948) for determining whether an organism is of significance in the rumen, in that it performs a chemical reaction known to occur there. However, it also gives rise to a considerable amount of hydrogen. Numerous authors have reported small quantities of hydrogen present in rumen gases, but Lugg (1938) in a very careful study found none at all. From the work of Barker (1936) we know that methane is produced by certain bacteria by the reduction of CO_2 with hydrogen. The mechanism of methane production in the rumen is unknown, but Elsden (private communication) has found indications that the reduction of CO_2 by hydrogen can take place *in vitro* with rumen contents. If *Veillonella* species are important in the rumen, they would provide hydrogen for methane production, and this would explain the lack of hydrogen in the rumen gases.

Phillipson (1942) found that the only natural diets to produce detectable amounts of lactate in rumen fermentation are mangolds, and, to a very small extent, oats. When lactate is formed in quantity as an intermediate in fermentation we should expect the conditions to be more favourable for growth of *Veillonella*. Changes in composition of the rumen flora have been observed to accompany changes in diet so that the only way of determining an organism's significance is to study it in the animal at different times of the year and under different feeding regimes. From counts made on winter grass in New Zealand it seems that *Veillonella* species are only of moderate importance but comparable counts of other species are not available for a definite decision to be made.

Elsden (1945) believed that members of the genus *Propionibacterium* were responsible for the formation of propionic acid in the rumen. It appears unlikely that they are important, as Delwiche (1948) and Johns (1949) have shown that members of this genus decarboxylate succinate only slowly, having a Q_{CO_2} of 1–4 at an optimum pH of 5–5·2 which is far removed from the optimum pH value for this reaction with rumen contents. No true propionibacteria were isolated by the procedure used in the present investigations, but the incubation periods were far too short to allow growth of *Propionibacterium* colonies.

There may be a number of bacterial species that play a part in the formation of propionic acid in the rumen as suggested by the spread of the pH optimum curve in Fig. 1; but it appears that the decarboxylation of succinate by washed suspensions of rumen bacteria is rapid enough to explain the propionic acid formation observed (Johns, 1948). It is probable that the dominant organism responsible will vary with the diet.

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The Mechanism of Propionic Acid Formation by *Veillonella gazogenes*

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SUMMARY: The mechanism of propionic acid formation from lactate by *Veillonella gazogenes* is according to the scheme:

lactate → pyruvate $\xrightarrow{CO_2}$ oxaloacetate → malate → fumarate → succinate → propionate + CO_2

The evidence for this scheme is: (*a*) Washed suspensions of the organism grown on lactate attack, under anaerobic conditions, pyruvate, oxalacetate, L-malate, fumarate and succinate, but not D-tartrate. (*b*) Organisms grown on D-tartrate attack it and all the substances listed in (*a*) except lactate. (*c*) Succinic acid is quantitatively decarboxylated to propionic acid and carbon dioxide. (*d*) The amount of propionic acid produced from lactate is influenced by the CO_2 concentration in the medium. (*e*) CO_2 is fixed in the carboxyl group of propionic acid during fermentation of lactate.

In a previous paper (Johns, 1951) the isolation of a strictly anaerobic micrococcus from the rumen of sheep was described. It was identified as *Veillonella gazogenes* and the products of its fermentation of lactate shown to be propionic and acetic acids, carbon dioxide and hydrogen. Unlike *Propionibacterium* it does not ferment sugars or form succinic acid as an end product.

The mechanism of propionic acid formation, which has been studied only in *Propionibacterium* has remained uncertain (Werkman & Wood, 1942; Krebs & Eggleston, 1941). Since lactate and pyruvate are readily converted to propionate by these bacteria it has been assumed that lactate is an intermediate in the reduction of pyruvate to propionate as follows:

$$CH_3.CO.COOH \xrightarrow{2H} CH_3.CHOH.COOH \xrightarrow{2H} CH_3.CH_2.COOH + H_2O.$$

Barker & Lipmann (1944), using dried cells of *P. pentosaceum*, ruled out this possibility by showing that, in the presence of sodium fluoride, lactate reduction could be completely blocked with only 57 % inhibition of pyruvate fermentation. Moreover, when the lactate reduction was blocked, no accumulation of lactate occurred when pyruvate was fermented.

The experiments of Carson & Ruben (1940) and of Wood, Werkman, Hemingway & Nier (1940) with isotopic carbon showed that propionic acid formed in the fermentation of glycerol by *Propionibacterium* contained fixed carbon dioxide. Wood, Werkman, Hemingway & Nier (1941) found this fixed carbon to be exclusively in the carboxyl group of the propionate, and also in the carboxyl groups of succinate. The quantitative results indicated that the most likely mechanism for propionic acid production was by decarboxylation of succinic acid. However, Werkman & Wood (1942) stated 'There is some evidence that propionic acid bacteria can decarboxylate succinate anaerobically but it is questionable whether or not the rate of this reaction is high enough to be of any considerable importance.' A number of workers have been unable to demonstrate any decarboxylation of succinate. The discovery that *Veillonella gazogenes* produces propionic acid offered an opportunity for the investigation of this problem in another bacterial species.

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EXPERIMENTAL

Methods

A strain of *V. gazogenes* isolated from the rumen of sheep was studied. The methods used in this work were as previously described (Johns, 1951) with the addition that washed suspensions of *V. gazogenes* were prepared and their metabolism studied by the Warburg technique. Bacteria were grown in litre flasks on the same medium as used for their isolation, with the addition of sodium sulphide to 0·01 % (w/v). Good growth was obtained in 12–18 hr.

The preparation of active washed suspensions of the bacteria was best accomplished by centrifuging down the cells and washing them with boiled distilled water containing 0·01 % (w/v) sodium sulphide. Much less active suspensions were obtained when glutathione was used as reducing agent. The Q_{CO_2} found with succinate as substrate was at least ten times greater in the presence of 0·01 % Na_2S than in its absence.

Pyruvic acid was estimated manometrically by the carboxylase method of Westerkamp (1933) as modified by Krebs & Johnson (1937).

Dry weights of bacteria in suspensions were determined by means of a photoelectric turbidimeter which had been calibrated against suspensions containing known dry weights of bacteria. In expressing the rate of a fermentation the conventional quotient 'Q' for gas production is employed. Q_{CO_2} is defined as μl. CO_2/mg. dry-weight bacteria/hr.

Preparation of $Na_2C^{13}O_3$. $KC^{13}N$ (18·8 atoms % excess) was converted to barium carbonate by combustion in the Pregl microcombustion apparatus and the CO_2 absorbed in saturated barium hydroxide. The precipitated barium carbonate was centrifuged down and carefully washed. The labelled carbon dioxide was then released by addition of acid in an evacuated vessel and absorbed in sodium hydroxide, thus yielding sodium carbonate containing isotopic carbon.

Carbon dioxide fixation experiment with $C^{13}O_2$. A growth experiment with *Veillonella* was carried out in a sealed tube. The inorganic medium +1·5 ml. sodium lactate (0·3 g./ml.) +0·4 % Difco yeast extract was adjusted to such a pH value that on the addition of 2 ml. of N-Na_2CO_3 the final pH was 7. The medium was autoclaved in the tube closed by a cotton-wool plug and the enriched C^{13} carbonate autoclaved separately. Before inoculation the labelled sodium carbonate was added to the medium. After inoculation from a 24 hr. culture of *Veillonella*, the cotton-wool plug was pushed down in the tube which was sealed off above the plug. Thus, as the fermentation proceeded, the gas produced was under pressure. As it had been shown previously that the amount of propionic acid formed was dependent on the partial pressure of CO_2 it was thought that, by having the gas under pressure, a greater proportion of propionic acid would be formed and the fixation of CO_2, if any, would be more convincingly demonstrated. A control fermentation in which no carbonate was added and the tube left unsealed was inoculated at the same time.

When the fermentation was completed, the volatile acids were distilled off, separated on a partition chromatogram and finally isolated as barium salts. To determine the total C^{13} in the acetic and propionic acids these barium salts were combusted in the Pregl micro-combustion apparatus and the carbon dioxide produced absorbed in barium hydroxide. The barium carbonate was dried for assay.

To determine the C^{13} concentration in the carboxyl group of the propionic acid, advantage was taken of the fact that when barium salts of lower fatty acids are heated at a sufficiently high temperature the corresponding ketone and carbon dioxide are produced, the carbon dioxide coming solely from the carboxyl group. In the dry distillation of barium propionate, for example, diethylketone and carbonate are formed:

$$(CH_3CH_2CO_2)_2Ba \rightarrow (CH_3CH_2)_2CO + BaCO_3.$$

According to the accepted mechanism of the reaction 50 % of the carboxyl carbon should be in the carbonate and the other 50 % in the ketone. This distribution of carboxyl carbon was confirmed by Wood, Werkman, Hemingway, Nier & Stuckwisch (1941) who used synthetic propionic acid labelled in the carboxyl group. They found that the reaction proceeded quantitatively at 460°.

A sample of barium propionate produced from the *Veillonella* fermentation was heated to 460°, the diethylketone driven off and the carbon dioxide liberated from the barium carbonate that remained and absorbed in barium hydroxide. The resulting barium carbonate was washed and dried. The samples of barium carbonate were assayed with the mass spectrograph for C^{13} excess over the normal ratio of C^{13}/C^{12} of 0·0109.

RESULTS

Succinic acid decarboxylation

To test whether *Veillonella* decarboxylated succinate, washed suspensions suspended in 0·1 M phosphate buffer (pH 6·8) were used and the manometers gassed with nitrogen (oxygen-free) and equilibrated at 37°. On tipping sodium succinate from the side bulb a rapid evolution of gas took place.

A balance experiment was carried out under the same conditions to identify the products formed. Gas production was measured with manometers and a large-scale fermentation was carried out in duplicate in Krebs vessels with the same proportions of constituents as in the manometers. Carbon dioxide was identified by using 20 % NaOH in the centre well of the manometer flasks. In the flasks where the CO_2 was absorbed it was shown that reaction had taken place by distilling 1 ml. of the liquid in a Markham steam distillation apparatus, collecting 30 ml. of distillate, and titrating the total volatile acid.

The propionic acid from the Krebs vessels was identified by its rate of movement down a silica gel chromatographic column and compared with that of a mixture of known acids. Only one band appeared as the product of the reaction, its identity being confirmed by adding pure propionic acid and observing that with this mixture again only one band appeared on the chromatogram. The results of a typical experiment are shown in Table 1. The theoretical figures given in Table 1 are calculated for the reaction:

$$HOOC.CH_2.CH_2.COOH \rightarrow CH_3.CH_2.COOH + CO_2.$$

The results are seen to agree very well with this equation.

Table 1. *Products of the decarboxylation of sodium succinate by washed suspensions of* Veillonella

Gas production measured in Warburg manometers containing 1·1 ml. 0·1 M phosphate buffer (pH 6·8) 0·2 ml. 2N-H_2SO_4; 1 ml. bacterial suspension (24 mg. dry-wt. bacteria); 0·2 ml. 0·1 M succinate.

Acid formation determined in Krebs vessels using same proportion of constituents. Temp. 37°.

	Substrate	Products (μl. CO_2)	Theoretical*
(*a*) Gas production	0·2 ml. 0·1 M succinate	445, 440	448
		mmol. propionic acid	
(*b*) Acid production	5·0 ml. 0·1 M succinate	0·505, 0·51	0·5

* Calculated for reaction $HOOC.CH_2CH_2.COOH \rightarrow CH_3.CH_2.COOH + CO_2$.

It was found that washed suspensions of the bacteria did not attack malonate, nor did malonate up to 0·01 M inhibit the decarboxylation of succinic acid.

The pH optimum for the decarboxylation reaction was determined using 0·1 M phosphate buffer at different pH values (checked by the glass electrode). The curve obtained by plotting μl. CO_2 produced in 5 min. against pH is shown in Fig. 1. The pH optimum of the decarboxylation reaction was between 5·9 and 6·0, with a Q_{CO_2} of 280. This is of the same order of activity as Gale's amino-acid decarboxylases with similar pH optima (Gale, 1946). Attempts to obtain a cell-free extract of the bacteria which should be active in the decarboxylation of succinic acid were unsuccessful.

Fermentation of possible intermediates in propionic acid formation

The action of washed suspensions on other possible intermediates in the formation of propionic acid was tested; these intermediates included malic, fumaric, oxaloacetic and pyruvic acids (Krebs & Eggleston, 1941). The manometric technique was used and the production of hydrogen looked for by the use of CO_2 absorbers in the centre well of the manometer flask. In all cases there was a rapid decomposition of substrate as shown by the production of gas. Only the L-isomer of malic acid was attacked.

Balance experiments were carried out with L-malate, fumarate and pyruvate as substrates. Table 2 shows the results obtained. The redox index was calculated by the method of Johnson, Peterson & Fred (1931). With pyruvate under

Table 2. *Products obtained from various possible intermediates in propionic acid formation by* Veillonella, *using washed suspensions*

Gas production measured in Warburg manometers and acid formation in Krebs vessels using the same proportion of constituents. Temp. 37°. Results expressed as mmol./100 mmol. substrate.

Substrate	Fermentation products (mmol./100 mmol. substrate) Propionic acid	Acetic acid	CO_2	H_2	Carbon recovery (%)	Redox index
Pyruvate	5·0	97	94	94	106	1·00
L-malate	62	32	150	32	100	1·02
Fumarate	66	31	152	31	103	1·03
Succinate	101	—	98	—	100	1·06

the conditions used the reaction was almost entirely a dissimilation of pyruvate into acetic acid, carbon dioxide and hydrogen, with only a small amount of propionic acid. As malate and fumarate are at the same level of oxidation it is to be expected that they would produce the products of fermentation in the same relative proportions. It was found that organisms grown on lactate as substrate did not attack D-tartrate in washed suspension. However, when *Veillonella* was grown on D-tartrate the washed suspension attacked D-tartaric acid with great vigour, the Q_{CO_2} being 400. This is illustrated in Fig. 2.

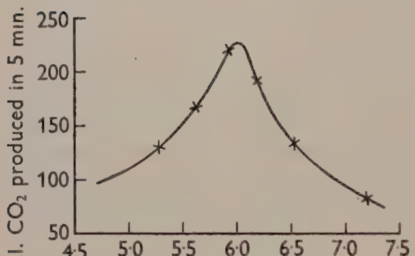

Fig. 1

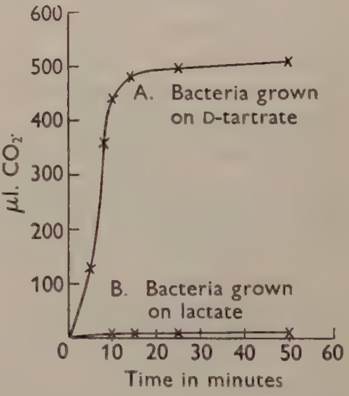

Fig. 2

Fig. 1. The decarboxylation of succinic acid by *Veillonella gazogenes* of various pH values. *V. gazogenes* 10 mg. dry-wt./vessel; 0·1 M phosphate buffer, 0·2 ml.; 2 N-H_2SO_4, 0·2 ml.; 0·1 M succinate. Temp. 36°. Duplicate manometers, in one, acid tip at beginning; in the other, acid tip after 5 min.

Fig. 2. Action on D-tartrate of washed suspensions of *V. gazogenes* grown (A) in presence of D tartrate, (B) in presence of lactate. Equal dry-weight/ml. of bacterial suspension used; 0·2 ml. 0·1 M substrate; 0·1 M phosphate buffer (pH 5·8); temperature 37°.

It was also found that organisms grown on D-tartrate quantitatively decarboxylated succinic acid and dissimilated pyruvate, oxaloacetate, L-malic and fumaric acids, but not lactate. The enzymes responsible for the fermentation of D-tartaric acid were thus adaptive, whereas those responsible for the dissimilation of oxaloacetic, L-malic, fumaric and succinic were constitutive.

Effect of an atmosphere of hydrogen on gas production

Cardon & Barker (1947) isolated an organism, *Diplococcus glycinophilus*, which formed acetic acid, carbon dioxide and hydrogen from glycine. They found that the amount of hydrogen produced depended on the partial pressure of hydrogen in the gas phase; in an atmosphere containing more than 25 % hydrogen, no hydrogen accumulated. Also Kubowitz (1934) found that the production of acetic acid, butyric acid, carbon dioxide and hydrogen in glucose fermentation by *Clostridium butylicum* was inhibited by hydrogen, lactic acid being formed instead.

To see whether an atmosphere of hydrogen made any difference to the amount of gas produced from pyruvate by *Veillonella* an experiment was run in which one set of manometers was filled with nitrogen and a duplicate set filled with hydrogen. The hydrogen production was determined with 20 % NaOH in the centre well of the manometer cups. Though hydrogen production was slightly less in an atmosphere of hydrogen the difference was hardly significant when compared with the major effects noted by Kubowitz (1934), and by Cardon & Barker (1947). The washed suspensions of *Veillonella* were inactive against sodium formate, indicating that hydrogen production was not via formic acid.

Hydrogenase. Experiments with washed suspensions in Thunberg tubes showed that methylene blue was rapidly decolorized in an atmosphere of hydrogen, indicating the presence of hydrogenase in the bacteria.

Pyruvate dissimilation

The optimum pH for the fermentation of sodium pyruvate was determined in Warburg manometers by measuring the hydrogen production at various pH values in 0·1 M phosphate buffer, CO_2 being absorbed by 20 % NaOH. As will be seen from Fig. 3 the pH optimum was about 6·2 and the $Q_{H_2}=114$. This pH optimum is considerably higher than that found for the fermentation of pyruvate by *Propionibacterium* (Barker & Lipmann, 1944). Woods & Clifton (1937) found a Q_{H_2} of 25–30 for hydrogen production by *Clostridium tetanomorphum* from pyruvate.

Fermentation of lactate by washed suspensions

Washed suspensions prepared by the method described above were often only feebly active against lactate. In a typical experiment 512 μl. of gas were formed from 0·2 ml. of 0·06 M pyruvate in 60 min. compared with 5 μl. from 0·2 ml. of 0·1 M lactate in the same period.

Generally it was found that the fermentation of lactate proceeded much better in bicarbonate buffer and an atmosphere of CO_2 than in phosphate buffer at the same pH and an atmosphere of nitrogen. On several occasions when there was no activity against lactate with washed suspensions in phosphate buffer, the reaction proceeded in bicarbonate. A typical result is shown in Fig. 4.

Fumarate was added in catalytic amounts (100 µg./vessel) to see whether it would act as a hydrogen acceptor and initiate the reaction. In flasks in which CO_2 was absorbed there was only a slight evolution of hydrogen which soon ceased; the reaction proceeded satisfactorily, however, in flasks which had no NaOH in the centre well and therefore CO_2 in the gas phase.

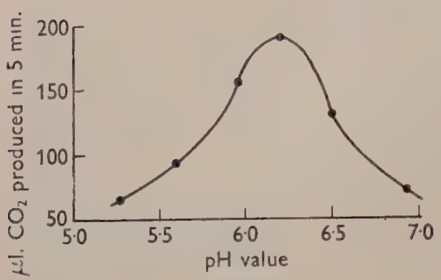

Fig. 3

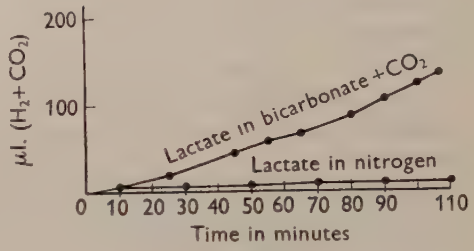

Fig. 4

Fig. 3. The rate of pyruvate dissimilation by washed suspensions of *Veillonella* as measured by H_2 production, at different pH values.

Fig. 4. The effect of the presence and absence of CO_2 on the rate of dissimilation of lactate by washed suspensions of *Veillonella*. The manometers gassed with nitrogen contained phosphate buffer at the same pH as the bicarbonate, pH 6·8.

Effect of CO_2 *concentration on fermentation of lactate*

As Elsden (1938) had shown with *Escherichia coli* that the amount of succinic acid formed in fermentation of pyruvate, glucose and galactose was altered by the carbon dioxide concentration in the medium, it was thought that if propionic acid were formed via decarboxylation of succinic acid, the CO_2 concentration should influence the amount of propionic acid formed.

To test this three Krebs vessels were shaken in a water-bath at 37°; each vessel contained the same volume of liquid, sodium lactate solution, washed suspension of bacteria, and were all at pH 6·8. Vessel 1 contained 0·1 M phosphate buffer and nitrogen (CO_2 and oxygen free) was bubbled through the medium continuously during incubation to remove CO_2 as it was formed. Vessel 2 contained 0·1 M phosphate buffer and was gassed with nitrogen at the start of the experiment but not during it, so that any CO_2 produced could accumulate. Vessel 3 contained bicarbonate buffer at pH 6·8 through which CO_2 (oxygen-free) was bubbled continuously during the fermentation. The vessels were thoroughly gassed with their respective gases before the substrate was added. The result was that while no lactate in vessel 1 disappeared, in vessels 2 and 3 all the lactate was utilized and the following propionic:acetic acid ratios were obtained, namely in vessel 2, 1·21:1 and in vessel 3, 2:1. From these results it will be seen that the manometric experiments with lactate in which CO_2 was absorbed were verified, i.e. fermentation did not take place in the absence of CO_2, and that the amount of propionic acid formed was dependent on the carbon CO_2 concentration in the medium. The experiment was repeated several times and in no case did fermentation occur when CO_2-

free nitrogen was bubbled vigorously through the fermentation liquid. The results indicated fairly strongly that formation of propionic acid follows the scheme proposed by Carson, Foster, Ruben & Barker (1941) and by Krebs & Eggleston (1941) for the formation of succinic acid, with the addition that propionic acid is formed by decarboxylation of succinic acid (see Fig. 5).

The further evidence which seemed desirable to justify this hypothesis appeared to be the demonstration of CO_2 fixation, and the location of the fixed carbon in the carboxyl group of the propionic acid formed. It was felt that the most satisfactory way to show CO_2 fixation in propionic acid would be by a growth experiment in the presence of $Na_2C^{13}O_3$ with sodium lactate as substrate. The results are given in Table 3. The 0·01 atom percentage excess in the acetic acid is not regarded as significant. The chromatographic method does not give complete separation of the bands (Barker & Wikén, 1948).

Table 3. *Results of* C^{13} *assay with mass spectrograph of acetic and propionic acids produced in* Veillonella *fermentation of lactate in the presence of* C^{13} *enriched sodium carbonate*

Each result is the arithmetic mean of at least ten determinations.

Sample	Atoms % C^{13}	Atoms % excess C^{13}
Cylinder CO_2	1·10	—
Acetic acid	1·11	0·01
Cylinder CO_2	1·10	—
Total propionic acid	1·78	0·68
Cylinder CO_2	1·10	—
Carboxyl of propionic acid	2·75	1·65

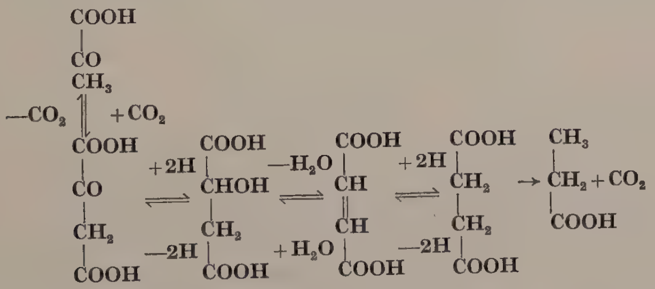

Fig. 5. Proposed mechanism of propionic acid formation from pyruvate.

There is definite evidence that CO_2 was fixed in the propionic acid formed. If the tracer carbon were located solely in the carboxyl group, the C^{13} excess in the carboxyl should be three times that in the whole molecule; in fact it was not quite three times. This can be explained by a slight mistake in the manipulation of material in the preparation of the barium carbonate; unfortunately the author did not have the opportunity to repeat the experiment. Despite these inaccuracies the results indicate that no CO_2 was fixed in the acetic acid and that it was fixed in the carboxyl group of propionic acid.

The ratio propionic acid: acetic acid in the fermentation carried out under pressure with sodium carbonate added was 1·9:1, whereas the control tube without carbonate and not under pressure was 1·19:1. All the lactate was fermented in each case. This confirms, in a growth experiment, the results already obtained with washed suspensions, namely, that the amount of propionic acid formed is dependent on the amount of CO_2 present.

DISCUSSION

The scheme set out in Fig. 5 appears to be supported for *Veillonella* by the following evidence: (*a*) During the fermentation of lactic acid, CO_2 is fixed in the carboxyl group of propionic acid. (*b*) All the postulated intermediates are attacked by the bacteria. (*c*) The occurrence of the reversible reactions from pyruvic acid to succinic acid are well established in other micro-organisms (Krebs, 1943) and in animal tissues (Utter & Wood, 1945). (*d*) Fermentation of lactate by washed suspensions of *Veillonella* did not take place in the absence of CO_2. (*e*) The amount of propionic acid formed depended on the CO_2 concentration in the medium. From the nature of the experimental conditions it was not possible to state whether all the propionic acid arose from CO_2 fixation. (*f*) Only bacteria which were grown on D-tartrate attacked this compound in washed suspension. The dicarboxylic acids of very similar structure, oxaloacetate, L-malate, fumarate and succinate, were fermented by bacteria grown on lactate or D-tartrate. This indicated that the enzymes which attack the postulated intermediates in the formation of propionic acid are truly constitutive, whereas those responsible for the primary breakdown of D-tartrate are adaptive. The fact that bacteria grown on D-tartrate did not attack lactate, shows that no propionate is formed by the direct reduction of lactate, confirming the findings of Barker & Lipmann (1944) in sodium fluoride inhibition studies with *Propionibacterium pentosaceum*.

Two mechanisms of hydrogen formation have been established with micro-organisms: (*a*) bacteria of the coli-aerogenes group form hydrogen by the decomposition of formate (Stephenson & Stickland, 1932, 1933; Kalnitsky & Werkman, 1943); (*b*) Koepsell, Johnson & Meek (1944) have shown that *Clostridium butylicum* will convert pyruvate anaerobically to acetic acid, CO_2 and H_2, formic acid not being an intermedate in this reaction.

With *Veillonella* it is thought that hydrogen production takes place by method (*b*) since it was shown that: (1) the bacteria do not attack formate; (2) in the fermentation of pyruvate, acetic acid, CO_2 and H_2 were formed in approximately equal quantities (Table 2); (3) the fermentation of L-malate and fumarate produced equal amounts of hydrogen and acetic acid. These findings seem to fit well with the idea that CO_2, H_2 and acetic acid arise in equimolar proportions from pyruvate as precursor. However, contrary to the findings of Kubowitz (1934) with *Clostridium butylicum* and of Cardon & Barker (1946) with *Diplococcus glycinophilus*, it was found that an atmosphere of hydrogen had no effect on the amount of hydrogen produced by *Veillonella* from pyruvate.

In addition to the formation of acetic acid from pyruvic acid it has been claimed that a direct splitting of succinic acid into two molecules of acetic acid can take place (Slade & Werkman, 1943). There seems to be no question of acetic acid being formed by this method in the present instance, for when succinic acid was attacked in washed suspensions only propionic acid and CO_2 were formed, and in the CO_2 fixation experiments significant amounts of isotopic carbon were not found in the acetic acid formed from the fermentation of lactate. If acetic acid had arisen from succinic acid, carboxyl-labelled acetic acid would have been expected to appear.

The fermentation of tartrate by bacteria has been little studied. Barker (1936) studied the fermentation of tartaric, fumaric and L-malic acids by *Aerobacter aerogenes* and found that they give rise to the same products as glucose. The probable course of the reaction is via oxaloacetic acid followed by decarboxylation to CO_2 and pyruvic acid (Stephenson, 1939). Propionic acid would be formed via L-malic, fumaric and succinic acids.

The anabolic (biosynthetic) process from pyruvate to carbohydrate has usually been conceived as the reverse of the catabolic process. This involves the production of phosphorylated derivatives of glucose, and the question arises, why is it that glucose is not fermented by some bacteria such as *Veillonella*? The so-called 'direct' fermentation of disaccharides also appears to be related to this problem. Pelczar & Doetsch (1949), when isolating and identifying bacteria of the genus *Neisseria* from the nasopharynx of humans, encountered several strains which fermented maltose with acid production while glucose was not fermented. It seems that in both cases the enzyme necessary to carry out the primary phosphorylation of the glucose may be missing.

My thanks are due to Dr S. R. Elsden and Dr R. Scarisbrick, for their interest and advice throughout this work, and to the late Dr Marjory Stephenson and Dr E. F. Gale for many useful discussions. My thanks are also due to Dr A. S. Macfarlane of the National Institute of Medical Research, Mill Hill, for the KCN enriched with C^{13} and to Mr Palmer of the Atomic Energy Research Establishment for carrying out the C^{13} assays.

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The Mechanism of Propionic Acid Formation by Propionibacteria

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SUMMARY: The main mechanism for propionic acid formation by organisms of the genus *Propionibacterium* is by the decarboxylation of succinic acid. The varying ratios of propionic to acetic acid reported in the literature as a result of the fermentation of glucose are attributed to differences in carbon dioxide tension and in the initial and final pH values of the cultures.

The method of formation of propionate from lactate and pyruvate by propionibacteria has remained uncertain. Werkman & Wood (1942) reviewed the various mechanisms suggested for the formation of propionic acid and made two proposals. The first was that water is removed from lactate to form acrylic acid which is then reduced to propionic acid. They regarded this as the most probable mechanism, though the reduction of acrylic acid by propionibacteria has not been demonstrated. The second possibility suggested was that the formation of propionic acid is by the decarboxylation of a symmetrical dicarboxylic acid. This was based on their experiments with isotopes and on unpublished evidence that the decarboxylation of succinate can occur by a reaction with a pH optimum of 5·2. Wood & Werkman (1942) conclude by saying: 'Frankly, sufficient data is not available with which to formulate a defensible scheme.'

In the present paper evidence is presented that the formation of propionic acid by the decarboxylation of succinate does occur in *Propionibacterium* as has been demonstrated with *Veillonella gazogenes* (Johns, 1951). Similar evidence, based on work with *Propionibacterium pentosaceum*, has been published by Delwiche (1948) since the completion of this work (Johns, 1948).

EXPERIMENTAL

Methods

Succinic acid was estimated by the procedure of Umbreit, Burris & Stauffer (1945); glucose by the method of Hassid (1937); volatile acids by the chromatographic method of Elsden (1946). The accuracy of the latter method for mixtures of acetic and propionic acids was found to be ±3 % with a satisfactory preparation of the silica gel. Lactic acid was estimated by the method of Friedemann & Graeser (1933). The general purposes salt mixture of Stephenson (1948) with 0·4 % Difco yeast extract was used as the basal medium for growing bacteria.

Organisms used. The strains of propionic acid bacteria used were *P. shermanii* E11.1 and *P. zeae* E8.1 kindly supplied by Prof. C. B. van Niel. A dried preparation of *P. shermanii* was made according to the method of Barker & Lipmann (1944).

RESULTS

P. zeae was grown at 30° for 40 hr. in the basal medium with 1 % (v/v) Na lactate solution (*c.* 50 %) added. The cells were spun down, washed twice with distilled water and taken up in phosphate buffer of pH 5·3. The results of the action of this suspension are shown in Table 1, which shows that there was an evolution of CO_2 (identified by using manometers with 20 % NaOH in the centre well) with succinate, but that the rate of evolution decreased rapidly with time.

Table 1. *Decarboxylation of succinate by washed suspensions of* Propionibacterium zeae *grown* 40 *hr. at* 30°

Bacterial suspension in buffer, 2 ml. (12 mg. dry weight/ml.), 0·2 ml. 0·1 M succinate; 0·5 ml. 0·1 M-phosphate buffer (pH 5·3) in side bulb with succinate.

Manometers ...	1	2	3	4
Period (min.)	Succinate (μl. CO_2)		Blank (μl. CO_2)	
5	15	16	7	8
20	39	41	20	20
60	64	68	22	23

Q_{CO_2} for first 5 min., 4·5; for first 20 min., 2·5; for first 60 min., 1·75.

Attempts were made to increase the rate of decarboxylation. It was thought that if cell permeability were a controlling factor dried cells might show an increased permeability to succinate. A dried preparation of *P. shermanii* was made from 10 l. of bacteria grown in 1 % (v/v) Na lactate + basal medium at 30° for 5 days. These cells were active with pyruvate, having a Q_{CO_2} of 2·5 compared with a Q_{CO_2} for fresh cells of 5·2, and kept well in a vacuum desiccator at 4°.

An experiment to determine the pH optimum of the decarboxylation reaction using 20 mg. dried bacteria/ml. is shown in Fig. 1. The pH optimum for CO_2 evolution was about pH 5 and the rate fell off sharply below this pH and above pH 6. The Q_{CO_2} at pH 5 was 0·35 as compared with a Q_{CO_2} of 0·48 with fresh cells.

The effect of detergents on the rate of succinate decarboxylation was tried with fresh cells. Neither an anionic (Aerosol O.T.) nor a cationic (CTAB) detergent over a wide range of concentrations increased decarboxylation but rather inhibited it. A third possibility for increasing the Q_{CO_2} when using succinate was the provision of a source of energy for the transference of succinate more rapidly across the cell wall; but rather than an increase in the rate of succinate utilization in the presence of pyruvate or lactate, there was a significant decrease. All efforts to prepare a cell-free extract which would decarboxylate succinate, from fresh or dried cells of *P. shermanii*, were unsuccessful.

It was difficult to carry out a balance experiment on the decarboxylation of succinate as the reaction proceeded so slowly that a long period of incubation or a large quantity of bacteria would be needed to obtain a reasonable conversion. It was decided that the easiest and most satisfactory method would be to carry out a growth experiment in which glucose and succinate together were fermented, with a fermentation of glucose alone as a control. In this case growth would result from the fermentation of glucose, the pH would fall and if the organisms were able to decarboxylate succinate they should do so at the acid pH.

Two identical manometers were used with 100 ml. flasks. The medium in both flasks was adjusted to pH 6·5 (by glass electrode); glucose was sterilized separately and identical amounts added to each flask. Both flasks were inoculated with 5 ml. of a 5-day culture of *P. shermanii* grown on sodium lactate. Samples were removed with sterile precautions for the initial determinations of glucose and succinate. The whole fermentation was carried out under sterile conditions in an atmosphere of nitrogen at 37°. Manometer readings were taken twice daily until gas production ceased. The two sets of manometer readings corrected to N.T.P. are plotted in Fig. 2.

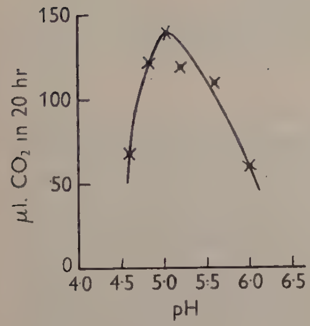

Fig. 1

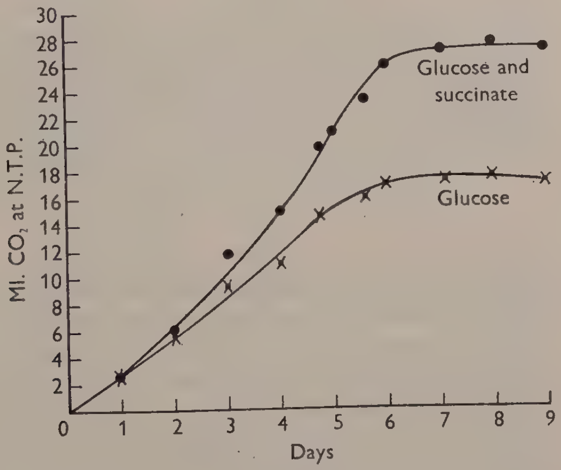

Fig. 2

Fig. 1. Rate of decarboxylation of succinic acid by dried cells of *P. shermanii* at different pH values.

Fig. 2. The effect of succinate on the production of CO_2 by *P. shermanii* grown with glucose.

When the fermentation had ceased the dissolved CO_2 in the fermentation liquid was determined by the method described by Peters & van Slyke (1932) for urine. The results are given in Table 2. The final acid figures include the amounts added in the inoculum; these were the same in both flasks. The 'initial succinate' in the glucose control *B* was that added with the inoculum. Neglecting the slight difference (0·02 mmol.) in the amount of glucose fermented and the acetic acid produced (0·01 mmol.), 0·48 mmol. of succinate disappeared to give rise to 0·51 mmol. of propionic acid and 0·46 mmol. of CO_2. The final pH was 4·4.

In spite of the large inoculum used it will be noticed from Fig. 2 that the complete fermentation of glucose took 6 days. This emphasizes the slow rate of

Table 2. *A comparison of the amount of* CO_2 *and propionic acid produced by* Propionibacterium shermanii *in the fermentation of: A, glucose + succinate; B, glucose alone*

Manometer *A*, 50 ml. basal medium (pH 6·5) and glucose + 1·5 ml. 0·1 M sodium succinate. Manometer *B*, the same except for 1·5 ml. distilled water in place of sodium succinate. Temp. 37°, gas phase N_2.

	A (mmol.)	*B* (mmol.)
Glucose fermented	1·32	1·30
Succinate fermented	0·49	0·01
Final propionate	2·76	2·25
Final acetate	0·76	0·75
Total CO_2	1·40	0·94
Excess propionate	0·51	—
Excess acetate	0·01	—
Excess CO_2	0·46	—

growth of the propionibacteria, and emphasizes the fact that it would not require a high rate of succinate decarboxylation to account for all the propionic acid produced. This experiment confirms the indications given by the washed suspension experiments that propionibacteria can decarboxylate succinate to propionic acid and carbon dioxide at pH values below 6.

The theoretical equation for the fermentation of glucose by propionibacteria is

$$3\ C_6H_{12}O_6 = 4\ C_2H_5COOH + 2\ CH_3.COOH + 2\ CO_2 + 2\ H_2O$$

giving a propionic acid:acetic acid ratio of 2:1. This ratio has rarely been observed. In a previous paper (Johns, 1950) it was demonstrated that the CO_2 concentration in the medium influenced the acetic to propionic acid ratio during the fermentation of lactate by *Veillonella gazogenes*. These experiments were repeated with *Propionibacterium shermanii* using three different concentrations of CO_2: *A* was carried out in a sealed tube with added sodium carbonate so that the fermentation finished up with CO_2 under pressure; *B* was similar to *A* but without sodium carbonate, not sealed, and incubated anaerobically; *C* was carried out in a 50 ml. volumetric flask to which was connected a second flask containing 20 ml. of alkaline pyrogallol to absorb the CO_2 produced during the experiment and to maintain anaerobic conditions. The results are shown in Table 3. As can be seen (from the results), there is

Table 3. *Effect of* CO_2 *concentration on the fermentation of lactate by* Propionibacterium shermanii

Incubation time 14 days at 30°; pH 7·2. Lactate 6 mmol. in each tube; 20 ml. basal medium. Acid production in mmol.

Tube ...	*A*, 2 mmol. Na_2CO_3 tube sealed	*B*, unsealed tube	*C*, CO_2 absorbed
Propionic acid (P)	3·45	3·38	3·38
Acetic acid (A)	1·61	1·72	1·78
Ratio P/A	2·14	1·96	1·90

a slightly higher ratio with the fermentation carried out in excess CO_2 as compared with that in which the CO_2 was absorbed, but the difference could hardly be considered convincing. In this experiment there was little change in acidity. Three molecules of lactic acid give rise to three molecules of volatile acid (two of propionic acid, one of acetic acid).

The above experiment was repeated with glucose as the substrate, when a considerable increase in acidity occurred. The results are presented in Table 4.

Table 4. *The effect of the* CO_2 *concentration on fermentation of glucose by* Propionibacterium shermanii

6 mmol. glucose in each flask; pH 6·5; temperature 30°, acid production measured in mmol.

	2 mmol. Na_2CO_3 tube sealed	Tube open	CO_2 absorbed
Propionic acid (P)	1·14	1·00	1·04
Acetic acid (A)	0·32	0·35	0·59
Ratio P/A	3·56	2·80	1·76

It will be seen in Table 4 that the increase in CO_2 tension led to a higher propionic:acetic ratio in the fermentation of glucose. No doubt this is in part the explanation of the widely varying ratios of propionic:acetic reported in the literature. The experiments of previous investigators have been carried out under varying conditions of pH and with different types of buffer.

DISCUSSION

Lactate and pyruvate are readily converted into propionate by propionibacteria, hence it was generally assumed (Kluyver, 1931) that lactate was an intermediate in the reduction of pyruvate, i.e. pyruvate $\xrightarrow{2H}$ lactate $\xrightarrow{2H}$ propionate. Wood & Werkman (1936) introduced the concept of heterotrophic utilization of CO_2 in connexion with their studies on the fermentation of glycerol by propionibacteria. Wood & Werkman (1938) proposed that succinic acid was formed in the fermentation by the combination of a C_1 and a C_3 compound. Krebs & Eggleston (1941) showed that the set of reactions from oxaloacetate to succinate occurred in the propionibacteria (oxalacetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate $\rightarrow$ succinate). Carson & Ruben (1940) using C^{11} and Wood, Werkman, Hemingway & Nier (1940, 1941) using C^{13}, confirmed that fixation occurred and that the labelled CO_2 appeared in the carboxyl group of succinic acid. However, quite unexpectedly, labelled carbon was also found in the carboxyl group of propionic acid. Both Carson, Foster, Ruben & Barker (1941), and Krebs & Eggleston (1941) explained the presence of isotopic carbon in propionic acid by postulating that the fixation process proceeded by the same pathway as for the formation of succinic acid but only as far as fumaric acid, and that by a reversal of these steps propionic acid was formed by reduction of the pyruvic acid which then contained the labelled carbon in its carboxyl group. Neither groups of workers demonstrated any decarboxylation of succinate. Barker & Lipmann (1944), working with dry preparations of *P. pentosaceum*, found, on the basis of fluoride inhibition studies, that lactate was not on the normal pathway from pyruvate to propionate.

The quantitative studies of Werkman & Wood (1942), in which they found equal concentrations of C^{13} in the carboxyl groups of the succinic and propionic acids, find their simplest interpretation in the presence of a succinic decarboxylase in the bacteria. The results presented above, and those of Delwiche (1948), demonstrate that propionibacteria are able to decarboxylate succinic acid below a pH of 6·0 at a rate sufficient to justify the assumption that this reaction is concerned in the production of propionic acid. Shaw & Sherman (1923) and Hitchner (1934) claimed to have demonstrated the fermentation of succinate with propionibacteria, but in both cases the utilization of succinate was only achieved under aerobic conditions. The experiments of Fromageot & Bost (1938) with washed suspensions of *P. pentosaceum* indicated that succinate was attacked only in the presence of glucose, but Krebs & Eggleston (1941) could not confirm this. The results of Fromageot & Bost (1938) are readily explained by the fact that the fermentation of glucose lowered the pH sufficiently to permit succinic decarboxylase activity. Krebs & Eggleston (1941) used bicarbonate buffer where a marked drop in pH does not take place. In the present work lactate or pyruvate in addition to succinate gave no increase in the rate of CO_2 evolution from succinate.

Although the equations for the fermentation of lactate (Fitz, 1878, 1879, 1880), and glucose by propionibacteria, have been established for a long time the ratio of 2:1 for propionic:acetic acid has rarely been obtained. The explanations for this variability in volatile acid ratio have previously centred round hypothetical pathways for anaerobic utilization of acetate (Wood & Werkman, 1938). Krebs & Eggleston (1941) rejected Wood & Werkman's hypothesis and suggested that acetate was oxidized to CO_2, but produced no convincing evidence to support this. Carson (1948) obtained indications in isotope experiments with propionibacteria that acetate was metabolized through a condensation reaction which subsequently yielded CO_2. The degree of importance of this reaction depended on the state of oxidation of the substrate. The amount of initial C^{14} acetate which was converted to $C^{14}O_2$ was 2 % with glycerol as substrate and 33 % with pyruvate.

If the main method of propionic acid formation is by succinate decarboxylation, it would be expected that the amount of propionic acid present would be dependent on the concentration of CO_2 in the medium. Elsden (1938) showed with *Escherichia coli* that the amount of succinic acid formed in the fermentation of pyruvate, glucose and galactose was influenced by the CO_2 concentration in the medium.

With propionibacteria a large effect of CO_2 concentration on the propionic: acetic ratio was only found with a substrate whose dissimilation caused an increase in acidity and so allowed the extra succinic acid formed in the presence of CO_2 to be decarboxylated.

There is always a tendency to obtain a higher propionic:acetic ratio with glucose than with lactate, e.g. in the present work the highest ratio with lactate was 2·14, while with glucose it was 3·56. As glucose and lactate are at the same redox level this difference in propionic:acetic ratio cannot be explained by the difference in the amounts of acetate converted to CO_2 due to their different levels of oxidation as indicated by the experiments of Carson (1948).

It appears that the large variations reported in the literature for the ratio of propionic acid:acetic acid in fermentations of glucose and lactate by propionibacteria can be explained on differences in CO_2 concentration in the medium. Evidently succinic acid can pass out of the bacterial cell at pH values above *c.* 6·5 but cannot re-enter until the pH drops below that level. When the CO_2 concentration in the medium is increased, more succinic acid is formed, the excess passes out into the medium and can only be decarboxylated when the pH falls giving a high propionic: acetic ratio.

Van Niel (1928) found that the propionic:acetic ratio was always less than 2 in lactate fermentations with propionibacteria, and always greater than 2 with glucose as substrate. The explanation seems to be that he absorbed the CO_2 produced in his lactate fermentations, and added $CaCO_3$ to neutralize the acid produced during the glucose dissimilation.

Foote, Fred & Peterson (1930), in glucose fermentation experiments with propionibacteria, did not allow the pH to fall, but neutralized the acidity with NaOH daily. The highest propionic:acetic ratio was 2·16 and large amounts of succinic acid accumulated. In one case $CaCO_3$ was used in the medium instead of the daily neutralization and this gave a propionic:acetic ratio of 2·85. Wood & Werkman (1934), in an experiment on glucose fermentation with $NaHCO_3$ as buffer, analysed the fermentation liquid at intervals. The pH remained constant at 7·2 until just before the end of the experimental period of 14 days. The ratio of propionic to acetic acid remained constant at about 2·4 during this time, while the amount of succinic acid steadily increased. Wood & Werkman (1936) used $CaCO_3$ as the buffer and found that the pH dropped from 7·0 to 5·5 during the fermentation of glucose, the amount of succinic acid formed decreased and the propionic:acetic ratio steadily increased.

Delwiche (1948) made an analysis of a fermentation broth for volatile acids at varying times and pH intervals throughout the fermentation. Significant amounts of propionic acid did not appear until the pH was below 6·5 and the peak of production was at a pH below 6. The ratio of propionic to acetic acid rose steadily from 0·7:1 at 23 hr. to 2·35 :1 at 41 hr. and there was no indication of a utilization of acetate of any period.

There were indications in the present work that there may be strain differences in the propionic:acetic acid ratio produced under the same conditions; this was indicated by the work of van Niel (1928).

The factors which appear to influence the propionic:acetic acid ratio in fermentation of glucose by propionibacteria may be summarized as follows: (*a*) the CO_2 tension in the medium; (*b*) the initial and final pH of the medium, the initial pH influencing the amount of succinic acid formed and the final pH determining how much will be decarboxylated; (*c*) the strain of *Propionibacterium*; (*d*) with the more oxidized substrates such as pyruvate the conversion of acetate to CO_2 evidently becomes important (Carson, 1948).

It seems quite clear therefore that in at least two bacterial species, *Propioni-*

bacterium and *Veillonella gazogenes*, the main mechanism of propionic acid production is by the decarboxylation of succinic acid. Whether there is a second mechanism for propionic acid formation in bacterial fermentations is not yet clear. Cardon & Barker (1947) studied an anerobic alanine-fermenting bacterium, *Clostridium propionicum*, which produces acetic acid, propionic acid and CO_2 from alanine and lactate. It rapidly converts acrylate to propionate (which *Propionibacterium* does not), and is inactive against malate and fumarate. It would be of interest to determine whether this organism fixes CO_2 in propionic acid.

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The Ability of Single Phage Particles to Form Plaques and to Multiply in Liquid Cultures

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SUMMARY: The results of testing a bacteriophage to a strain of clover nodule bacteria using young (1 day) and old (5 days) bacterial cultures both fit to the hypothesis that phage multiplication can be initiated by single phage particles. As the same phage preparations gave more plaques on solid media and higher proportions of liquid cultures in which phage multiplication could be detected, with young than with old bacterial cultures, the fit to the hypothesis is not an evidence that every single phage particle will multiply. It may be so when young bacterial cultures are used, although there is no positive evidence for it. With older bacterial cultures definitely only a proportion of viable phage particles succeed in starting phage multiplication, the proportion decreasing with the increasing age of bacterial cultures used for testing.

The two methods mainly used for quantitative estimation of bacteriophage are plaque counts and serial dilution in liquid media. These methods are satisfactory for estimating relative phage activities in different preparations of the same phage, but they are also used to estimate absolute numbers of phage particles. The translation of results obtained by either of the two methods into numbers of phage particles is based on the assumption, originally made by d'Herelle (1917, 1920, 1926), that a single phage particle placed in a medium containing susceptible bacteria always causes general clearing in a liquid medium or local clearing (a plaque) on an agar medium. This assumption is often taken as self-evident, although observations have been recorded that make it doubtful.

Bronfenbrenner & Korb (1925), who worked with a phage to '*Bacillus dysenteriae*', found that estimates of numbers of phage particles by the plaque count were below those obtained by the dilution method. Similarly, Ellis & Delbrück (1939) found that the estimates of numbers of a coli phage from plaque counts were about 40 % of those estimated from the proportion of liquid cultures with demonstrable phage multiplication. Bronfenbrenner & Korb (1925) also found that factors such as concentration of agar and the age of bacterial culture influence numbers of plaques. Clark (1927) showed mathematically that the assumption of a single phage particle being always sufficient to cause lysis in a liquid bacterial culture is not compatible with observed average proportion of parallel titrations giving the same dilution end-point.

Most workers translate the numbers of plaques directly into numbers of phage particles. Those who doubted the validity of this procedure, judged its efficiency by comparing the results with those obtained from liquid cultures, which were assumed to give a correct estimate of the numbers of phage particles, although this point was not established. The work described in this paper was done to test the value of both methods. Experiments were so designed that the results could be examined statistically for their compatibility with the assumption that single phage particles initiate multiplication in liquid cultures, irrespective of whether it is followed by obvious lysis of the culture, and could be compared with those obtained by the plaque-count method.

MATERIALS AND METHODS

Experimental. Clover nodule bacteria (*Rhizobium trifolii*, strain 317) were used as a host and the bacteriophage was obtained from soil as previously described (Kleczkowska, 1945) and isolated from a single plaque. Phage cultures referred to as 'stock cultures' were lysed liquid bacterial cultures passed through a Chamberland L3 filter. The media used were those used by Kleczkowska (1945).

Plaques were obtained by the poured plate method of Kleczkowska (1945). One volume of a phage preparation diluted in sterile liquid medium was mixed with nine volumes of a liquid bacterial culture, and 1 ml. of the mixture was mixed with 10 ml. of molten and cooled (to 42°) 0·7 % agar medium, which was then poured into a Petri dish. The dilutions of phage preparations given in the text are the final dilutions in the liquid bacterial cultures, so that the dilution of a preparation is the fraction of 1 ml. of the preparation introduced into a plate. Plaques were counted after 48 hr. incubation at 25°.

The liquid bacterial cultures used for plating were obtained by inoculating a loopful from an agar culture into 9 ml. of sterile liquid medium, which was then incubated at 25° for 24 hr. unless otherwise stated.

In experiments with liquid bacterial cultures, each tube containing 9 ml. of the culture was inoculated with 1 ml. of a phage preparation diluted in sterile liquid medium and 1 ml. of the sterile medium without phage was added to a control tube. The dilutions of phage preparations recorded are dilutions in the sterile medium, so that the dilution means the fraction of 1 ml. of an undiluted preparation inoculated into the bacterial culture.

The bacterial cultures used in any one experiment were prepared by adding a drop from a suspension to a series of tubes each containing 9 ml. of sterile medium. The tubes were incubated at 25° for lengths of time given in the text and then inoculated with the phage. After such inoculation, the tubes were incubated for 24 hr. at 25° and then for 5 days at room temperature, during which time they were periodically inspected.

All the tubes that were not clearly lysed were tested for the presence of phage by placing a drop of their contents on the surface of an agar plate. Normal bacterial growth was taken as evidence that no phage was present. Two controls were run simultaneously with the test. In one a drop of the tested culture was placed on the surface of the agar plate and a drop of a phage culture added to it. The presence of bacterial growth here would mean either that a bacterial contamination of the culture had occurred or that a phage-resistant bacterial variant had appeared. In the other control a drop of a normal culture of host bacteria was placed on the surface of the plate. This was done to provide a standard of comparison for normal bacterial growth. The method can be expected to detect phage only when present in such concentrations that it is expected to occur in each drop, and when contamination with other bacteria has not occurred and no phage-resistant variant has appeared.

Statistical. The distribution of numbers of plaques obtained on different plates inoculated with the same dilution of a phage preparation is later shown to be Poissonian. Therefore, if N is the number of plates and M is the mean number of plaques per plate, M/N is an estimate of the variance of the mean.

The probability p_i of the phage occurring in a detectable quantity in a liquid bacterial culture inoculated with a given phage preparation at a dilution, will be shown to be

$$p_i=1-e^{-z_i\lambda}.$$

The value of the constant λ can be estimated by the method of maximum likelihood, and, consequently, the variance of the estimate can also be obtained.

Let n be the number of liquid bacterial cultures inoculated with each of m different dilutions of a phage preparation. Let $z_1, z_2, \ldots, z_m$ be the dilutions and $r_1, r_2, \ldots, r_m$, respectively, the number of cultures in which the presence of phage was detected. Putting $p_i=1-e^{-z_i\lambda}$ and $q_i=e^{-z_i\lambda}$, the probability of obtaining the values of r's that were actually obtained would be

$$\phi=\prod_{i=1}^{m}\left[\frac{n!}{r_i!(n-r_i)!}p_i^{r_i}q_i^{(n-r_i)}\right]. \qquad (1)$$

To obtain the estimate of λ by the method of maximum likelihood, the value of λ that makes the value of ϕ a maximum, must be chosen. This is done by solving for λ the equation

$$\frac{\partial}{\partial\lambda}\log\phi=\sum_{i=1}^{m}\left[\frac{r_i-n(1-e^{-z_i\lambda})}{1-e^{-z_i\lambda}}z_i\right]=0. \qquad (2)$$

The variance, σ^2, of the estimate of λ, if large samples are used, is then obtained from the equation

$$-\frac{1}{\sigma^2}=E\left(\frac{\partial^2}{\partial\lambda^2}\log\phi\right)=-E\left[\sum_{i=1}^{m}\frac{z_i^2r_iq_i}{p_i^2}\right]=-\sum_{i=1}^{m}\frac{z_i^2nq_i}{p_i},$$

so that

$$\sigma^2=\frac{1}{n\sum_{i=1}^{m}\left[z_i^2e^{-z_i\lambda}/(1-e^{-z_i\lambda})\right]}. \qquad (3)$$

If only one dilution, z, of a phage preparation is used, the equation (2) for obtaining the estimate of λ, becomes

$$r/n=1-e^{-z\lambda}, \qquad (4)$$

and the equation (3) for the variance becomes

$$\sigma^2=\frac{1-e^{-z\lambda}}{z^2ne^{-z\lambda}}. \qquad (5)$$

If estimates of λ and also mean numbers of plaques per plate, are based on samples of reasonable size, they both can be considered to be approximately normally distributed estimates of some unknown existing values. As a variance can be assigned to any of these estimates, the standard deviation of a difference between any two estimates can be computed and the statistical level of significance of the difference assessed.

RESULTS

Inoculation of agar plates and liquid bacterial cultures with the same phage inoculum

Each of 200 agar plates and of 100 tubes containing a 24 hr. bacterial culture, was inoculated with 1 ml. of a phage stock culture diluted 10^{-9} in the 24 hr. liquid bacterial culture.

The mean number of plaques per plate was 1·655. Table 1 shows the observed distribution of plaque numbers on the plates and also the expected Poisson distribution with the mean equal to 1·655. The value of the χ^2 for the deviations of observed from expected numbers of plates shows that the deviations are not significant; (the value of P being about 0·5). It can be assumed, therefore, that the distribution of plaque numbers is Poissonian, as was found by Ellis & Delbrück (1939) with a coli bacteriophage.

Table 1. *The distribution of plaque numbers*

The mean number of plaques per plate: 1·655.

No. of plaques	Observed nos. of plates		Expected nos. of plates		χ^2 (D.F. = 4)
0	45		38·22		1·2028
1	58		63·26		0·4374
2	51		52·34		0·0344
3	28		28·88		0·0267
4	10		11·94		0·3149
5	3		3·96		
6	3		1·10		
7	2	8	0·26	5·36	1·3004
8	0		—		
9	0		—		
	200				3·3166

The Poissonian character of the distribution of plaque numbers can be considered as evidence that plaques are formed by single-phage particles. (Any aggregate of phage particles that is able to infect a bacterial cell as effectively as a single particle is considered as one particle.) However, only a proportion, say ρ, of particles able to form plaques may do so. If μ is the mean number of phage particles in 1 ml. of the inoculum, the mean number of plaques per plate would then be an estimate of $\rho\mu$.

Evidence is given below that multiplication of phage in a liquid bacterial culture can be initiated by a single phage particle. It is again possible that only a proportion, say ρ', of particles able to start multiplication, do so. If so, the proportion of cultures in which phage multiplication will be expected to occur, will be $p = 1 - e^{-\rho'\mu}$.

Among the 100 liquid cultures inoculated with the same inoculum as the agar plates, phage multiplication occurred in 85, all of which were lysed. Thus $0{\cdot}85 = 1 - e^{-\rho'\mu}$, so that $\rho'\mu = 1{\cdot}897$. It will be noticed that $\rho'\mu$ corresponds to λ of the equation (4) (with $z = 1$), so that the variance of the estimate of $\rho'\mu$ is given by the equation (5) and equals 0·0567.

Thus 1·655 is an estimate of $\rho\mu$ and 1·897 of $\rho'\mu$, with variances 0·0083 and 0·0567, respectively. The standard deviation of the difference between the two estimates is $\surd(0{\cdot}0083 + 0{\cdot}0567) = 0{\cdot}255$. The ratio of the difference to its standard deviation, $0{\cdot}242/0{\cdot}255 = 0{\cdot}95$, corresponds to the value of P of about 0·35, so that the difference is not statistically significant. ρ and ρ' may be equal, but their value is unknown. In other words, in the prevailing experimental conditions a similar proportion of phage particles both formed plaques and lysed liquid cultures. Every phage particle may have multiplied, but this is not established.

Effect of age of bacterial culture on the numbers of plaques

If only a proportion of phage particles form plaques, the proportion may vary with varying experimental conditions. To see whether age of bacterial culture influences the proportion, a phage stock culture was plated at a dilution of 10^{-7} with liquid bacterial cultures of three different ages: 1, 3 and 5 days. Ten plates were inoculated with the culture of each age. The mean numbers of plaques per plate were, respectively, 110·9, 98·7 and 88·9. Taking the variances of these means as equal to 1/10 of their values, the difference between any two of the three means is statistically significant. Thus the older the bacterial culture the smaller was the proportion of phage particles that formed plaques. The size of plaques also decreased with the increased age of the bacterial culture. In all these respects the phage used in this work behaved similarly to a Shiga phage used by Bronfenbrenner & Korb (1925).

Relationship between phage concentrations and the proportions of liquid bacterial cultures with demonstrable phage multiplication

Tables 2 and 3 show the results of four experiments, in each of which eight different dilutions of a phage preparation were made and 100 liquid bacterial cultures were inoculated with each dilution. The four experiments were made at different times using different phage-stock cultures. The stock cultures were diluted 10^{-8}, and the values of z_i given in the tables and in the text below show further dilutions of this. The tables record the numbers of cultures that were lysed and also the numbers of those that did not lyse but in which the presence of phage was detected by the method described above. The sums of the two numbers give the total numbers of cultures in which phage had multiplied. Unless the phage introduced in the inoculum had multiplied, its presence would be undetectable by the method.

In two experiments, 2-day, and in the other two experiments, 5-day bacterial cultures were used. The younger cultures were slightly opalescent and contained about 2×10^7 bacterial cells per ml.; the older cultures were strongly opalescent, contained about 2×10^8 bacterial cells per ml. and some slimy sediment.

Simultaneously with each of the four experiments with liquid cultures, the phage stock cultures were also plated at a dilution 10^{-8} using 24 hr. bacterial cultures. The mean numbers of plaques per plate obtained on eight plates are given in the tables.

Let μ be the mean number of phage particles in 1 ml. of a phage stock culture diluted 10^{-8}. If it be assumed that one phage particle can start phage multiplication in a liquid bacterial culture and that ρ is the proportion of particles that will start multiplication in a given set of experimental conditions, the expected number of cultures in which the phage will multiply, would be

$$Y_i = 100(1 - e^{-z_i \rho\mu}), \tag{6}$$

where z_i is further dilution of the phage culture as defined above. $\rho\mu$ corresponds to λ of the equations (1)–(3), so that the estimate of $\rho\mu$ by the method of maximum likelihood is obtained by solving the equation (2) for λ. If the assumptions on which the equation (6) is based are correct, the variance of the estimate of $\rho\mu$ will be given by the equation (3).

Tables 2 and 3 show the expected numbers of cultures in which phage had multiplied, computed by substitution into the equation (6) of the values of $\rho\mu$ obtained by the method of maximum likelihood. Tables 2 and 3 also show that for all the four experiments the values of χ^2 obtained from deviations of observed from the expected numbers are not significant. The assumptions on which equation (6) is based, can, therefore, be assumed to be correct.

The values of the χ^2 were computed according to the formula

$$\chi^2 = \Sigma \left(\frac{x_i^2}{Y_i} + \frac{x_i^2}{100 - Y_i} \right),$$

where Y_i's are the expected numbers of cultures where phage multiplication occurred, and x_i's are deviations of observed from the expected numbers. If, for a given phage dilution, the value of Y_i or of $(100-Y_i)$ was smaller than about 5, the values of Y_i, of $(100-Y_i)$ and of the corresponding observed numbers for two or more different phage dilutions were pooled, and the curly brackets in Tables 2 and 3 show where this was done. Each phage dilution, or group of pooled data, contributed one degree of freedom and there was a loss of one degree of freedom for the estimation of $\rho\mu$. Results obtained with some phage dilutions, with which all 100 cultures were expected to and did show phage growth, did not contribute to the estimation of $\rho\mu$ and were not used for computation of the χ^2.

One purpose of the experiments shown in Tables 2 and 3 was to find if the value of ρ varied with the age of bacterial cultures. This is not shown by comparing the values of $\rho\mu$ obtained in the experiments with the younger and the older cultures. The large scale of these experiments did not allow them to be run simultaneously, so that the value of μ could have varied from one experiment to another. However, the phage preparations used in each experiment were also tested on agar plates, using 24 hr. bacterial cultures for plating and keeping other experimental details as constant as possible.

Table 2. *Results of inoculating 2-day liquid bacterial cultures with differently diluted phage preparations*

	Liquid bacterial cultures								
			Observed nos. of cultures						
	$\rho\mu$	Dilution z_i	Lysed	Non-lysed, phage present	Total phage present	Expected no. of cultures with phage present	χ^2	Plaque count $\rho'\mu$	Difference between $\rho\mu$ and $\rho'\mu$
Exp. A	19·1	1·0	100	—	100	100·00	—	16·5	Difference = 2·6
	(variance =	0·5	100	—	100	100·00	—	(variance =	S.D. = $\sqrt{(1·643 + 2·065)} = 1·925$
	1·643)	$0·5^2$	99	1	100	99·16	0·0988	2·065)	
		$0·5^3$	89	0	89	90·81			
		$0·5^4$	65	0	65	69·69	1·0413		
		$0·5^5$	44	3	47	44·95	0·1698		$\frac{\text{Diff.}}{\text{S.D.}} = 1·35$
		$0·5^6$	29	3	32	25·80	2·0080		
		$0·5^7$	10	2	12	13·86	0·2898		
							3·6077		$P = 0·18$
							(D.F. = 4; $P = 0·4$)		
Exp. B	18·7	$0·5^2$	99	0	99	99·07	1·9392	22	Difference = 3·3
	(variance =	$0·5^3$	86	0	86	90·34		(variance =	S.D. = $\sqrt{(1·502 + 2·75)} = 2·062$
	1·502)	$0·5^4$	65	1	66	68·93	0·4008	2·75)	
		$0·5^5$	44	1	45	44·25	0·0228		
		$0·5^6$	34	0	34	25·34	3·9641		$\frac{\text{Diff.}}{\text{S.D.}} = 1·60$
		$0·5^7$	13	1	14	13·59	0·0143		
		$0·5^8$	9	2	11	7·05	2·8519		
		$0·5^9$	4	1	5	3·59			
							9·1931		$P = 0·11$.
							(D.F. = 5; $P = 0·1$)		

Table 3. *Results of inoculating 5-day liquid bacterial cultures with differently diluted phage preparations*

	Liquid bacterial cultures								
			Observed nos. of cultures						
	$\rho\mu$	Dilution z_i	Lysed	Non-lysed, phage present	Total phage present	Expected no. of cultures with phage present	χ^2	Plaque count $\rho'\mu$	Difference between $\rho\mu$ and $\rho'\mu$
Exp. C	4·35	0·1	12	18	30	35·31	1·235	11·75	Diff. = 7·4
	(variance =	$0{\cdot}1\times0{\cdot}5$	10	9	19	19·55	0·019	(variance =	S.D. = $\sqrt{(0{\cdot}253+1{\cdot}469)}=1{\cdot}312$
	0·253)	$0{\cdot}1\times0{\cdot}5^2$	5	7	12	10·30	0·313	1·469)	
		$0{\cdot}1\times0{\cdot}5^3$	2	5	7	5·29	3·130		
		$0{\cdot}1\times0{\cdot}5^4$	1	3	4	2·68			
		$0{\cdot}1\times0{\cdot}5^5$	0	2	2	1·35			$\frac{\text{Diff.}}{\text{S.D.}}=5{\cdot}64$;
		$0{\cdot}1\times0{\cdot}5^6$	0	2	2	0·68			
		$0{\cdot}1\times0{\cdot}5^7$	0	1	1	0·34			
							5·281 (D.F. = 4; $P=0{\cdot}25$)		$P<0{\cdot}001$.
Exp. D	11·3	1·0	93	7	100	100·00	—	19·0	Diff. = 7·7
	(variance =	0·5	69	28	97	99·65	3·663	(variance =	S.D. = $\sqrt{(0{\cdot}559+2{\cdot}375)}=1{\cdot}713$
	0·559)	$0{\cdot}5^2$	71	21	92	94·07		2·375)	
		$0{\cdot}5^3$	40	38	78	75·65			
		$0{\cdot}5^4$	28	24	52	50·65	0·300		
		$0{\cdot}5^5$	17	19	36	29·75	0·073		$\frac{\text{Diff.}}{\text{S.D.}}=4{\cdot}5$
		$0{\cdot}5^6$	5	15	20	16·18	1·869		
		$0{\cdot}5^7$	9	3	12	8·45	1·076		
							1·629		
							8·610 (D.F. = 5; $P=0{\cdot}15$)		$P<0{\cdot}001$

23·2

If ρ' is the proportion of phage particles that formed plaques, the mean number of plaques gives an estimate of $\rho'\mu$, and the value of μ, though unknown, is identical with that of μ of the corresponding experiment with the liquid cultures. The values of ρ and ρ' can, therefore, be compared by comparing the estimates of $\rho\mu$ and $\rho'\mu$.

It is not certain that the value of ρ' was constant in all tests, but it is assumed that it did not vary considerably. Thus the values of ρ for liquid bacterial cultures of different ages can be compared indirectly by comparing them with the values of ρ'.

Tables 2 and 3 give approximately statistical levels of significance of the differences between the estimates of $\rho\mu$ and of $\rho'\mu$. The estimates of the values of $\rho\mu$ for the young bacterial cultures (Table 2) did not differ significantly from the estimates of $\rho'\mu$. It is possible, therefore, that with the younger bacterial cultures $\rho = \rho'$, and their value may have been about 1·0, but no evidence can be offered in favour of any particular value.

The estimates of the values of $\rho\mu$ for the older bacterial cultures (Table 3) were significantly smaller than the estimates of $\rho'\mu$. For these cultures, therefore, $\rho < \rho'$, so that the values of ρ were definitely smaller than 1·0.

It is concluded that single phage particles can start multiplication in liquid bacterial cultures. When the cultures were 5 days old only a proportion of phage particles did this, and when younger cultures were used, the proportion was increased, but there is no evidence to show whether or not it reached 1·0 even under optimal conditions.

Tables 2 and 3 show that phage multiplication could occur without obvious lysis of the bacterial culture. This happened very rarely with the younger bacterial cultures (Table 2), but frequently with the older cultures (Table 3). The younger bacterial cultures became wholly clear when lysed, whereas the older cultures although obviously lysed were still somewhat opalescent.

DISCUSSION

The failure of some phage particles to multiply can have two possible meanings. If the phage population is homogeneous, each phage particle stands an equal chance of success, but the magnitude of the chance is determined by some conditions in the bacterial culture, such as heterogeneity of bacterial population, non-uniformity of bacterial surface or presence of inhibitory metabolic products in the medium. If the phage population is heterogeneous, a proportion of phage particles may be incapable of infecting, the magnitude of the proportion being determined by some conditions of the cell or of the surrounding medium. Experimental evidence of heterogeneity of phage populations, even after repeated isolations from single plaques, is supplied by the results obtained with a few coli phages by Andrewes & Elford (1933), who found that phage particles differed in their reactions with antisera, and by Burnet & Freeman (1937), who found that particles of a phage against *Bacillus dysenteriae* differed in their susceptibility to inactivating effect of a preparation obtained from an extract from host bacteria.

If the behaviour of bacteriophages is analogous to that of animal and plant viruses, the results obtained with a bacteriophage might help to interpret the results obtained in the experiments designed to find whether one virus particle is sufficient to start a local infection in an animal or a plant, or whether a minimal dose containing many virus particles is necessary.

An equation equivalent to the equation (6) could not be fitted satisfactorily to numbers of symptoms of local infections with papilloma and vaccinia viruses (Bryan & Beard, 1940) or with several plant viruses (Kleczkowski, 1950). The numbers could be fitted to the sigmoidal curve when logarithms of virus concentrations were plotted against proportions of inoculated susceptible regions of a host that developed local symptoms of infection. Bryan & Beard (1940) concluded from this that the hypothesis of a single virus particle being able to form a local symptom is false, and that minimal effective virus doses vary as a result of variation in regional host susceptibility in such a way that logarithms of minimal effective virus doses are normally distributed. Kleczkowski (1950) considered Bryan & Beard's conclusion as likely, but he drew attention to the fact that the hypothesis of single virus particles being able to form local lesions on plant leaves could be retained if an additional assumption were made that would make the value corresponding to ρ of the equation (6) vary from one susceptible region to another, and a number of such possible assumptions was suggested. The results obtained with bacteriophage suggest a likely auxiliary assumption.

Let us assume that symptoms of local infections in animals and plants can be caused by single virus particles, and, by analogy with bacteriophage, let the auxiliary assumption be made that there is a limited probability that a virus particle, when placed in a susceptible region of host tissue, will successfully initiate a process of local infection. If susceptible regions are not identical, the magnitude of the probability may vary from one region to another, and, therefore, a value corresponding to ρ of the equation (6) would not be constant within the same experiment, and this would account for the impossibility of fitting an equation similar to (6) to experimental results.

The equation (6) could be fitted to the results obtained when liquid bacterial cultures were inoculated with a variously diluted phage preparation, only because the value of ρ was constant within one experiment, and this was so because all the bacterial cultures used within one experiment resembled one another closely. If variety were introduced into bacterial cultures, for example, by using in the same experiment bacterial cultures of different ages, the value of ρ would not be constant but vary from one culture to another, and this would make it impossible to fit the equation (6) to experimental results. Therefore, the fact that an equation equivalent to (6) could not be fitted to experimental results obtained with some animal or plant viruses does not necessarily invalidate the hypothesis of one virus particle being able to initiate a local infection.

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An Antibiotic Assay Tray

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SUMMARY: The construction is described of a tray made of an age-hardening aluminium alloy and toughened glass for use in antibiotic assays on agar media.

A tray for holding agar medium, to be used in the assay of antibiotics, was designed with a view to hard wear in sterilization. The tray described below has stood up to sterilization in the autoclave over 100 times without casualty or visible deterioration and may therefore be considered satisfactory.

The trays consist of anodized frames (1) of an age-hardening aluminium alloy (see Fig. 1. for cross-section) with bottoms (2) of toughened glass ('Armour plate'). The glass plates fit *loosely* into a recess at underside of the frames and are held up by a number of powerful cup springs (3).

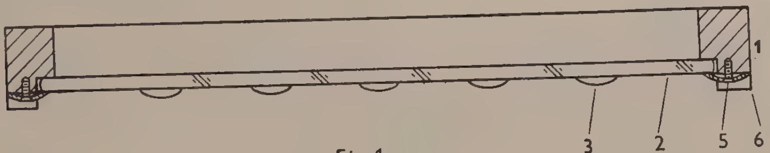

Fig. 1

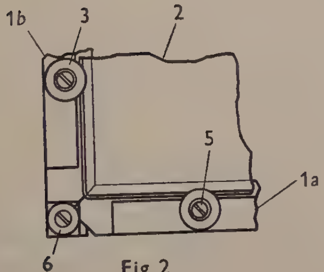

Fig. 2

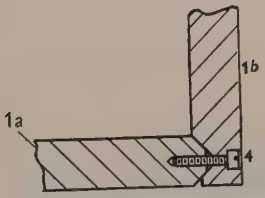

Fig. 3

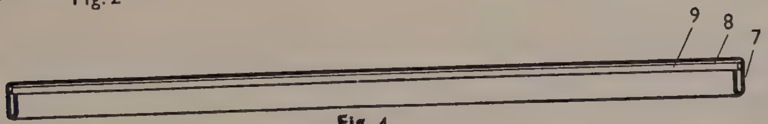

Fig. 4

Fig. 1–4

Figs. 2 and 3 illustrate the construction of the frame. Each is made up of two bars (1*a*) the ends of which are truncated 90° prisms and two bars (1*b*) with 90° Vee grooves near their ends. The prisms are pulled into the Vees by brass bolts (4). A recess is milled on the underside of the assembled frame. The ledges so formed against which the glass plates will be forced are thus all brought into one plane. After drilling and tapping holes for brass clamping screws (5) the frames are marked, taken apart and anodized. Toughened glass plates are unfortunately always somewhat warped. On final assembly it is therefore advisable to leave the corner bolts (4) slack at first, pull up the frame against the glass and finally tighten the corners. It will now be found that the frame is warped slightly. Small feet (6) of anodized aluminium are fitted to the corners and adjusted in height so that their free surfaces lie in a plane parallel to the top surface of the glass plates (2).

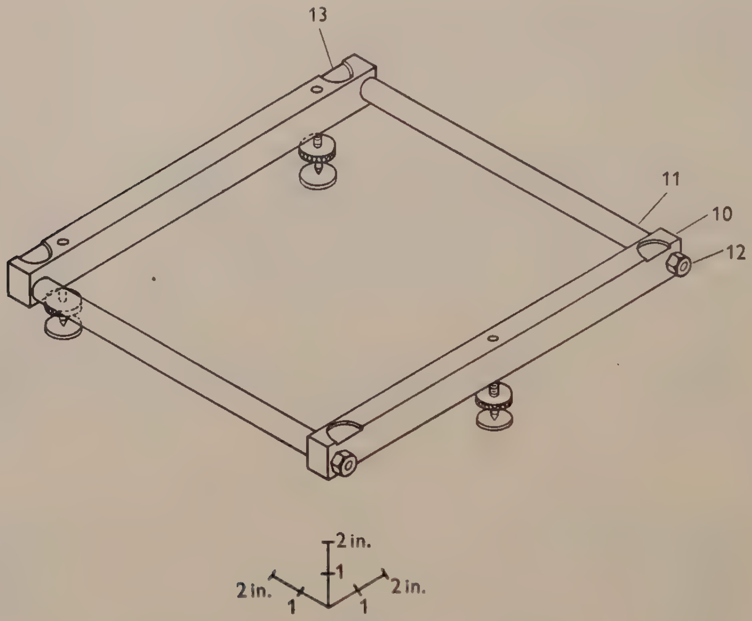

Fig. 5

Lids (7) are provided for each tray. They are folded from pure aluminium sheet as indicated in Fig. 4. Sheets (8) of filter-paper are held up by short pieces of stainless steel wire (9) (1/8 in. diameter) which are sprung into the recesses formed by upturning the aluminium sheet. The wires are arranged approximately 2 in. distant from one another.

Fig. 5 shows one of two levelling stands built up from aluminium alloy bar (10), brass tubing (11) and screwed rod (12). A tray is placed with its feet in the recesses (13) shown. The levelling stand is then adjusted until a spirit-level placed on the glass plate proves this to be horizontal. All the other trays will then be found to be levelled too when placed on the stand and after pouring agar medium will be in layers of uniform thickness.

The trays have been used for a number of different kinds of assay, in some of which antibiotic was incorporated in the agar. Since small amounts of agar are liable after cleaning to remain lodged in cracks between the glass and the frame, it is advantageous to be able to dismantle the trays for cleaning. This can be done by undoing the retaining cup springs.

A photograph of an actual assay of penicillin is shown in Pl. 1, (facing page 279) in which the tray holds sixty-four 'fish spine' beads. The use of these beads for holding the fluid to be assayed was suggested by G. A. Stewart and R. H. Thorp (private communication).

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Nitrification of Oxime Compounds by Heterotrophic Bacteria

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SUMMARY: Production of nitrite from the oxime of pyruvic acid was observed with three groups of heterotrophic soil bacteria: *Nocardia corallina, Alcaligenes* spp. and *Agrobacterium* spp. The first could nitrify up to 64 % of the oxime-nitrogen in an inorganic salts solution, the second and third up to 43 and 36 %, respectively. Glucose inhibited nitrification by stimulating the synthesis of cell substance; nitrification by *Nocardia corallina* ceased at C:N ratios above 20 :1. Approximately 75–90 % of the oxime-nitrogen was recovered as nitrite + cell-nitrogen in cultures of *Nocardia* and *Alcaligenes* spp. In peptone medium *Nocardia corallina* converted the oxime almost quantitatively to nitrite; the rate of nitrite production was parallel with that of cell multiplication. *Alcaligenes* and *Agrobacterium* spp. caused a further removal of the nitrite in peptone medium, the latter organism by denitrification. The oxime of oxaloacetic acid was readily nitrified by *Alcaligenes* spp. Acetoxime was only nitrified to a slight extent and free hydroxylamine apparently not at all. Several other organisms, of which *Corynebacterium equi* was a possible exception, did not nitrify pyruvic acid oxime.

Hydroxylamine and its aldehyde and ketone derivatives, the oximes, are known to arise in certain microbial processes. Endres (1935), Horner & Burk (1939), Aso, Migita & Ihda (1939), and Virtanen & Laine (1939) found small amounts of oxime in *Azotobacter* cultures grown with molecular nitrogen, nitrate or nitrite as source of nitrogen. Virtanen, Hakala & Järvinen (1949) reported that *Azotobacter* also forms oxime when utilizing ammonia. Virtanen & Laine (1939) found the oxime of oxaloacetic acid (oximinosuccinic acid) among the products excreted from root nodules of pea plants, together with small amounts of nitrite that were probably formed by decomposition of the oxime. Hydroxylamine, either as the free compound or as an oxime, has been detected as an intermediate in the reduction of nitrate and nitrite by bacteria other than *Azotobacter* (Blom, 1928; Lindsey & Rhines, 1932; Aubel, 1938), by yeast (Virtanen & Csáky, 1948), and by fungi (Steinberg, 1939). According to Steinberg, *Aspergillus niger* also produces hydroxylamine when growing on ammonia or urea, especially the latter. The formation of hydroxylamine and oximes may thus take place by oxidation of ammonia as well as by reduction of nitrate or nitrite. The utilization of these compounds by micro-organisms has been little studied. Free hydroxylamine is known to be toxic and has generally been found not to be metabolized at low (non-toxic) concentrations. It seems to be utilized to a slight extent by *A. niger* (Steinberg, 1939) and *Torulopsis* (Virtanen & Csáky, 1948), but its tendency to decompose spontaneously into ammonia and nitrogen makes the conclusion appear uncertain. Maurer (1928) observed reduction of pyruvic acid oxime to alanine by yeast. Virtanen (1938) stated that *Rhizobium* grows well with oxaloacetic acid oxime as source of nitrogen, and that sterile pea seedlings can utilize small concentrations of this oxime though not that of pyruvic acid. Novak & Wilson (1948) found that oxime-N was not available to *Azotobacter*, though acetaldoxime, according to Stapp & Ruschmann (1924), is toxic to this organism. Lees & Quastel (1946) showed that pyruvic acid oxime, but not hydroxylamine, is readily nitrified in soil, and Quastel & Scholefield (1949) adduced evidence that its conversion to nitrite is due to a previously unknown type of nitrifying bacterium, probably heterotrophic; isolation of such organisms was reported but no further details given. Jensen (1950) found that pure cultures of *Nitrosomonas* were unable to nitrify pyruvic acid oxime; a search for oxime-decomposing organisms has therefore been made.

METHODS

The oxime of pyruvic acid (α-oximinopropionic acid) was prepared by mixing solutions of hydroxylamine HCl and three to six (usually four) molar equivalents of sodium pyruvate at pH 7·5–8·0. The solution was sterilized by filtration (Berkefeld) and added aseptically to the autoclaved basal medium. Sterile solutions of oxime very rarely contained more than 0·1 μg. NO_2-N/ml. (maximum 0·25 μg./ml.) after incubation up to 2 weeks. The usual basal medium (referred to as medium A), was a solution of the following inorganic salts in tap water (all as %, w/v): K_2HPO_4, 0·05; $MgSO_4$, $7H_2O$, 0·02; NaCl, 0·05; $FeSO_4$, $7H_2O$, trace. In some cases nutrient broth or 0·5 % (w/v) Bactopeptone was used instead. Usually 2 ml. 0·025 M oxime solution were added to 8 ml. of basal medium in a 50 ml. flask (final concentration, 0·005 M oxime or 70 μg. oxime-N/ml.). Inoculum was given as a thin saline suspension of cells from 2-day-old agar cultures. Duplicate cultures were incubated at 25°.

Nitrite was determined colorimetrically by the Gries-Ilosvay method, as described by Snell & Snell (1945); the culture fluid was clarified with lead acetate and phosphate, or with aluminium sulphate and sodium hydroxide, when it was desired to determine nitrogen (by semi-micro Kjeldahl) in the precipitated cell substance. Glucose was determined by the micro-method of Stiles, Peterson & Fred (1926).

RESULTS

Isolation of organisms

Enrichment cultures of oxime-decomposing organisms were made by adding soil particles to medium A containing oxime, or by adding this medium to soil samples. After incubation for about 1 week, plate cultures were made on a similar medium solidified with agar. The agar medium showed a strong nitrite reaction after incubation for another week, and numerous bacterial colonies developed from which pure cultures were obtained by plating on nutrient agar. Three different kinds of bacteria were isolated which showed nitrification in quantitative tests.

Description of the oxime-nitrifying bacteria

The first organism was readily identified as *Nocardia corallina* (*Bergey's Manual*, 1948). Young cultures showed long, branching, Gram-positive rods, initially forming small but definite mycelia, later short rods and cocci, sometimes weakly acid-fast (10 sec., 5 % H_2SO_4). Abundant, pasty, pinkish growth on glucose-nutrient agar; no liquefaction of gelatin; no indole; no acid or gas from carbohydrates. Twenty-four strains isolated from an oxime-enriched clover soil from Odum Experimental Station (Jutland) all showed nitrification. But this is not a constant character of *N. corallina*; among six strains from non-enriched soils only two nitrified oxime.

The second organism had properties in agreement with the general description of *Alcaligenes* in *Bergey's Manual* (1948): small Gram-negative rods, after 3–4 days almost coccoid, mostly non-motile but somewhat motile in broth, and producing a scant and uncharacteristic whitish growth on nutrient agar. No liquefaction of gelatine; no indole; no acid or gas from carbohydrates; alkaline reaction in peptone media and in milk which otherwise shows no pronounced change. Nitrate is reduced to nitrite in medium A, and disappears rapidly from nutrient broth without any visible gas evolution. Three strains were isolated from a red loam soil from São Paulo, Brazil. These organisms seem most nearly to resemble *A. metalcaligenes* but differ by their motility in broth.

The third organism was a single strain from oxime-enriched soil (Odum Experimental Station) apparently belonging to the genus *Agrobacterium* (*Bergey's Manual*, 1948) and resembling *A. radiobacter* in most respects, but giving no characteristic growth on potato and in milk. Denitrification, with gas evolution, took place in peptone media but not in medium A; another similar culture isolated from a non-enriched soil failed to nitrify oxime.

Quantitative experiments were made with two strains of *Nocardia corallina* (K 3 and K 6), one *Alcaligenes* sp. (B 2), and the *Agrobacterium* sp. (K 1). None of these produced nitrite from ammonium sulphate (in Winogradsky's solution for *Nitrosomonas*, with or without addition of peptone), or from alanine or asparagine. Alanine (0·05 %) appeared to be inhibitory to *Alcaligenes* and *Agrobacterium*, but was an excellent source of nitrogen for *Nocardia*.

Nitrification of pyruvic acid oxime

Medium A with different pyruvate concentrations. One strain each of the three organisms were grown in medium A with 0·005 M hydroxylamine + 3, 4 or 6 molar equivalents of sodium pyruvate. All three showed vigorous nitrification (Table 1). *N. corallina* converted 50–60 % of the oxime-N to nitrite, the pyruvate concentration having little effect. *Agrobacterium* behaved somewhat erratically. *Alcaligenes* showed a decreasing formation of nitrite as the C:N

Table 1. *Effect of pyruvate concentration on nitrification of pyruvic acid oxime*

Organisms grown in medium A containing 0·005 M oxime

		NO_2-N (µg./ml.)					
		Nocardia (K3)		*Alcaligenes* (B2)		*Agrobacterium* (K1)	
Pyruvate (M)	C:N ratio	5 days	12 days	5 days	12 days	5 days	12 days
0·015	7·7	40	35	25	25	20	15
0·020	10·3	40	35	18	15	7·5	25
0·030	15·4	30	35	2·3	1·4	17·5	20

ratio increased, presumably because the increasing excess of available carbon permitted assimilation of more oxime-N into cell substance.

To test this point, *Alcaligenes* sp. was grown on a larger scale (25 ml. medium in 250 ml. Erlenmeyer flask). The bacterial cells were precipitated with aluminium hydroxide and nitrogen determined in the precipitate. Table 2 shows that the increase from three to four equivalents of pyruvate did result in an increased production of cell substance; no explanation was found for the still further decrease in nitrite production observed with six equivalents of pyruvate without further increase in cell-nitrogen. Estimation of residual oxime was not attempted, because this cannot be done accurately in the presence of nitrite (Csáky, 1948).

Table 2. *Production of nitrite and bacterial substance from pyruvic acid oxime by* Alcaligenes *sp.*

Medium A containing 0·005 M oxime and sodium pyruvate as indicated; incubation, 7 days

	Sodium pyruvate (M)		
	0·015	0·020	0·030
NO_2-N (µg./ml.)	30	15	2·3
N in bacterial cells (mg./25 ml.)	0·53	0·85	0·86
Oxime-N recovered as nitrite (%)	43	21	3·3
Oxime-N recovered as cell-nitrogen (%)	30	49	49·2
Oxime-N recovered total (%)	73	70	53

Influence of glucose on nitrification. The three organisms were grown in medium A containing oxime with four equivalents of pyruvate (as in all subsequent experiments) with and without 0·8 % glucose. After 10 days' incubation cell-nitrogen was determined in the precipitate formed with aluminium hydroxide, and nitrite and residual glucose in the filtrate. As shown in Table 3, glucose inhibited nitrification by stimulating cell synthesis. With *Nocardia* and *Alcaligenes* some 80–90 % of the oxime-N was recovered as nitrate + nitrogen in cell substance. The apparent inhibition of nitrification by glucose was complete with *Nocardia*, probably because this organism readily utilizes nitrite with glucose as a source of carbon.

Table 3. *Effect of glucose on production of nitrite and of bacterial substance from pyruvic acid oxime*

Organism stated in medium A with 0·005 M oxime; glucose 0·8 % (w/v) when added

	Nocardia (K3)		*Alcaligenes* (B2)		*Agrobacterium* (K1)	
Addition of glucose	−	+	−	+	−	+
NO_2-N (µg./ml.) after 3 days	20	0·3	8·5	7·5	7·5	0·4
NO_2-N (µg./ml.) after 6 days	30	0	20	6·0	12·5	1·5
NO_2-N (µg./ml.) after 10 days	45	0	20	7·5	25	0·4
After 10 days:						
Glucose consumed (%)		68		35		25
Oxime-N recovered						
As nitrite (%)	64	0	29	11	36	0·6
As cell-nitrogen (%)	26	90	55	72	10	42
Total (%)	90	90	84	83	46	43

N. corallina was grown in medium A (25 ml. in 250 ml. Erlenmeyer flasks) with the addition of different concentrations of glucose sterilized by filtration. The results (Table 4) show that nitrite accumulation ceased at C:N ratios above 20:1, and again some 80–90 % of the oxime-N was recovered.

Table 4. *Effect of variation of glucose concentration on production of nitrite and of cell substance from pyruvic acid oxime*

Nocardia corallina (K3) in medium A containing 0·005 M oxime; incubated for 8 days

	Glucose added (%)				
	0	0·05	0·10	0·20	0·80
C:N ratio	10·3	13·1	16·0	21·7	56·0
Glucose consumed (%)	—	100	100	100	43
NO_2-N (μg./ml.)	40	20	14	0	0
N in bacterial cells (mg./25 ml.)	0·41	0·93	1·04	1·35	1·41
Oxime-N recovered					
As nitrite (%)	57	29	20	0	0
As cell-nitrogen (%)	24	58	71	77	81
Total (%)	81	82	91	77	81

Nitrification in complex media. *N. corallina* showed vigorous nitrification in broth and peptone media, while the action of *Alcaligenes* and *Agrobacterium* was inconstant and mostly negative, apparently because the nitrite was further transformed by these organisms in such media. A quantitative test with *Nocardia corallina* (Table 5) showed practically complete nitrification of the oxime-N in sugar-free medium after 3 days, and some inhibitory effect of glucose, presumably owing to increased cell synthesis. The experiment was repeated with a smaller inoculum and with nitrite determinations at shorter time intervals; viable counts on nutrient agar plates were also done. Fig. 1 shows a very close correlation between nitrite accumulation and bacterial multiplication; approximately 90 % of the oxime was nitrified after 4 days. Apparently the nitrite was not utilized in the presence of peptone, but accumulated in the medium.

Table 5. *Effect of glucose on nitrification of pyruvic acid oxime in peptone medium*

Nocardia corallina in 0·4 % (w/v) Bactopeptone solution containing 0·005 M oxime; glucose 0·8 % (w/v) when added

	NO_2-N (μg./ml.)			
	N. corallina, K3		*N. corallina*, K6	
Period (days)	−(glucose)	+(glucose)	−(glucose)	+(glucose)
3	60	25	50	26
7	70	62	65	50
11	70	55	65	55

Nitrification of other oximes

Quastel & Scholefield (1949) found nitrification of oxaloacetic acid oxime, but not of acetoxime, in soil tests. An experiment (Table 6) was made with oxaloacetic acid oxime prepared from hydroxylamine and three molar equivalents of sodium oxaloacetate; this oxime was readily nitrified by *Alcaligenes*, but not by *Nocardia* and *Agrobacterium*.

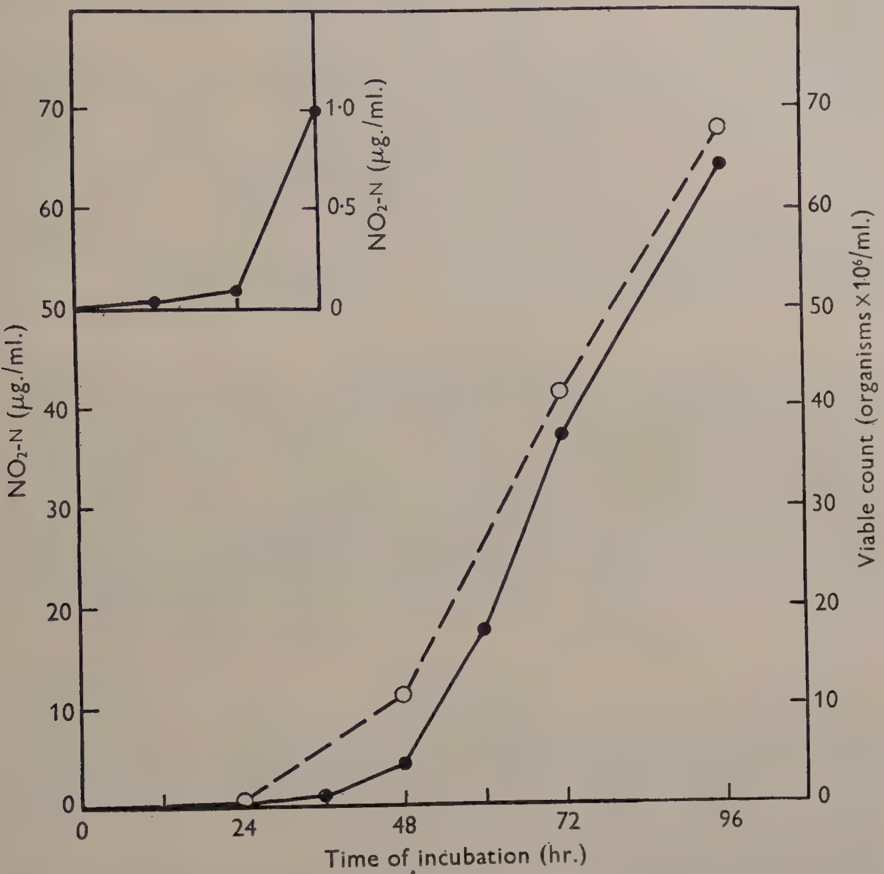

Fig. 1. Production of nitrite and multiplication of *Nocardia corallina* (K3) in 0·4 % (w/v) peptone solution (glucose-free) + 0·005 M pyruvic acid oxime. ●——●, NO_2-N; ○- - -○, viable count. Top left diagram: nitrite formation during first 36 hr. drawn to a larger scale.

Table 6. *Nitrification of oxaloacetic acid oxime*

Organism as stated in medium A containing 0·005 M oxime

	NO_2-N (µg./ml.) found			
Period (days)	*Alcaligenes* (B2)	*Nocardia* (K3)	*Agrobacterium* (K1)	Sterile control
5	30	0·05	0·05	0·05
10	30	0·02	(0)	0·05

Acetoxime (prepared from hydroxylamine and four or eight molar equivalents of acetone) was only slightly attacked by *Nocardia corallina* in peptone medium, where 3–5 % of the oxime-N was nitrified after 2 weeks. It seems improbable, indeed, that acetoxime would be altogether resistant to decomposition, since this compound is quite likely to arise in nature by association between denitrifying bacteria and *Clostridium acetobutylicum*. Blom (1928), for instance, found accumulation of oxime by addition of acetone to cultures of denitrifying bacteria.

Free hydroxylamine was strongly toxic. *Alcaligenes* sp. grew feebly in peptone solution with 0·005–0·02 % hydroxylamine HCl, but no nitrate appeared. Growth of *Nocardia corallina* was suppressed at these concentrations and visibly inhibited at 0·001–0·002 % (2–4 μg. hydroxylamine-N/ml.); the accumulation of nitrite-N did not exceed 0·4 μg./ml. and was hardly significantly higher than in the sterile control medium. Free hydroxylamine thus appears not to be nitrified, although the evidence is not conclusive in view of the instability of hydroxylamine, particularly in the presence of nitrite.

Tests with other organisms

A number of stock cultures (*Staphylococcus aureus*, *Bacillus subtilis*, *B. mycoides*, *Escherichia coli*, *Aerobacter aerogenes*, *Alcaligenes faecalis*, *A. metalcaligenes*, *Pseudomonas aeruginosa*, *Rhizobium* spp., *Agrobacterium radiobacter*, *Azotobacterium chroococcum*, *Mycobacterium phlei*, and various actinomycetes) failed to produce nitrite from pyruvic acid oxime in nutrient broth or in medium A. Only a strain of *Corynebacterium equi*, from the State Veterinary Serum Laboratory, Copenhagen, gave evidence of slight nitrification in peptone solution, and this did not exceed 3 % of the oxime-N after 2 weeks' incubation.

DISCUSSION

The experiments show clearly that several types of bacteria can decompose oximes with formation of nitrite and can utilize oxime-N for cell synthesis to an extent which largely depends on the supply of available carbon. Nitrite formation is probably not an energy-yielding process like ammonia oxidation by *Nitrosomonas*, but may tentatively be regarded as a hydrolytic process schematically expressed thus: $R.C(:NOH)+H_2O \rightarrow R.CH_2+HNO_2$. The nitrite found in the culture medium may thus represent unutilized nitrogen accumulating as a waste product when the organic radical serves as source of energy. In agreement with this view, *Nocardia* and *Alcqligenes* (but not *Agrobacterium*) were able to grow in medium A with sodium propionate and sodium nitrate as sources of carbon and nitrogen.

On the basis of these results it is possible to agree with the suggestion of Quastel & Scholefield (1949) that 'the process of nitrification may not be wholly accomplished by the autotrophic organisms'. Since hydroxylamine can definitely be formed by denitrification and other processes of nitrate and nitrite reduction, the occasional presence of oximes under natural conditions is quite likely. In such cases the oxime-decomposing bacteria might bring about a re-nitrification of nitrogen previously reduced from the nitrate or nitrite stage. A heterotrophic nitrification of oximes not resulting from nitrate reduction might take place in the rhizosphere of leguminous plants if nitrogenous compounds are excreted from the nodules (Virtanen, 1938). Such excretion, however, appears to be somewhat exceptional, and oxime-N rarely accounts for more than 1–2 % of the total excreted nitrogen (Virtanen & Laine, 1939). Also the quantities of oxime produced by other micro-organisms (e.g. *Azotobacter*) or by lower animals (Yamafuji, 1950) appear to be small. It thus appears doubtful whether the amounts of nitrite arising by this route would be quantitatively important in comparison with those produced from ammonia by *Nitrosomonas*. And in any case no organism other than the autotrophic *Nitrobacter* is yet known to accomplish the final stage of the whole nitrification process in soil, i.e. the oxidation of nitrite to nitrate.

My sincere thanks are due to Prof. E. J. Petersen, Royal Veterinary and Agricultural College, Copenhagen, for checking the cultural characters of *Alcaligenes* and *Agrobacterium*, to Mr H. Sorensen, B.Sc., for valuable assistance with the experimental work, and to Lovens Kemiske Fabrik, Ltd., Copenhagen, for a gift of oxaloacetic acid.

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A Re-investigation of the Root-Nodules of Species of *Elaeagnus, Hippophae, Alnus* and *Myrica,* with Special Reference to the Morphology and Life Histories of the Causative Organisms

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SUMMARY: Root-nodules of *Elaeagnus* spp., *Hippophae rhamnoides*, *Alnus glutinosa* and *Myrica gale* are modified lateral roots. The enlarged cortical cells of an actively growing nodule contain the endophyte in several stages of development. The apical meristem is free from infection but the cells just behind it show a plasmodial stage. In older parts of the nodules, spherical vesicles are formed in species of *Elaeagnus*, *Hippophae* and *Alnus* and club-shaped bodies in *Myrica* and from these small, bacteroid-like granules are produced. There is some evidence that granules formed in different host cells fuse in pairs and possess some ability to migrate within the nodule and into the soil. No definite hyphae can be seen in any of the nodules, but well-marked protoplasmic strands or lines of flow are present, particularly in the endophyte of *Myrica*.

The controversial views of earlier writers are discussed, and it is concluded that the nodule organisms are members of the Plasmodiophorales.

The conflicting opinions of earlier workers on the nature of the endophytes in the root-nodules of certain non-leguminous plants made a re-investigation desirable. Accordingly, nodules from roots of *Elaeagnus macrophylla* Thbg., *E. angustifolia* L. (*E. hortensis* M. Bieb), *Hippophae rhamnoides* L., *Alnus glutinosa* (L.) Gaertn. (*A. rotundifolia* Stokes) and *Myrica gale* L. were examined throughout the year by means of squash preparations, hand sections, and freezing-microtome sections of fresh material; and variously stained microtome sections of material fixed in Craf II (see Sass, 1940, p. 19) and embedded in paraffin wax. The most useful general stain was carbol fuchsin counterstained with light green. Aniline blue in lactic acid was also used. For cytological details gentian violet, alone or counterstained with light green, or iron alum haematoxylin, alone or similarly counterstained, were used. Other stain combinations were tried but gave no further information. Gram-staining gave variable results.

The observations of earlier workers (Servettaz, 1909) on the morphology of the nodules were confirmed. In all species examined the nodules are modified lateral roots. The swelling begins at the base of the young lateral and the nodule afterwards forks and may eventually produce a large compound gall. The young nodules have no root-hairs and are enclosed in a cork coat of two to three layers of cells. In *Myrica* the tips of the roots remain free from infection; they become cut off by the cork layer and are eventually sloughed off. When soil conditions are suitable the tips of the modified roots of the other species resume growth and break through the outer cork layer. They can then be seen as white tips to the branches of the nodules.

Elaeagnus macrophylla, E. angustifolia *and* Hippophae rhamnoides

In active nodules of these three species the endophyte is most conspicuous in the cortical cells just behind the growing apex. Here the infected cells are enlarged and filled with the endophyte. The infected lateral shows at least three zones corresponding with the stage of development of the parasite (Pl. 1, figs. 1, 2), and this may account for the varied descriptions met with in the literature. In the extreme tip of the nodule the host cells are not infected, are normal in shape and the walls are thin and entire (Fig. 1*a*). Just behind this uninfected growing tip the cortical cells are not completely filled with the endophyte which is seen as an amorphous mass of naked protoplasm surrounding the host nucleus (Fig. 1*b*, *c*). Further back from the tip, where the infection is presumably more advanced, some cells show the host nucleus pushed to one side with the remains of the host protoplast which is in process of absorption by the parasite (Fig. 2; Pl. 1, fig. 3). Other cortical cells are completely filled with an undifferentiated mass of protoplasm which resembles the plasmodium of *Plasmodiophora*. The nucleus of the host cell remains more or less intact for some time after the rest of the cell contents have been absorbed, but later becomes distorted and is finally also absorbed. Delicate protoplasmic strands which pass through the host cell walls connect the 'plasmodia' in neighbouring cells (Fig. 1*b*, *c*). Observations of this 'plasmodial' zone immediately behind the apical meristem of the infected root suggest that the endophyte advances with the seasonal growth in length of the nodule by penetrating the cell walls and becoming established in the young cells shortly after they are formed. The 'plasmodia' of the parasite appear to flow through the pierced wall of an infected cell into a younger and hitherto uninfected one, the nucleus of which is soon surrounded.

In the second and most conspicuous zone of the infected area, situated on the side of the plasmodial zone remote from the apex of the nodule, the majority of the host cells are each filled with a cluster of small spherical bodies (diam. about 4μ.) embedded in a network of protoplasm, derived from the plasmodium, which may be connected by protoplasmic strands with that in neighbouring cells (Fig. 1*f*; Fig. 2; Pl. 1, fig. 3). In some cells these bodies may be seen in process of differentiation. At this stage preparations stained with gentian violet or iron alum haematoxylin show numerous minute nuclei in process of division (Fig. 1*d*; Fig. 3; Pl. 1, fig. 4). These are so small that the method of division could not be determined with certainty, but the presence of an equatorial dark-stained band suggests a normal nuclear division. This division is simultaneous throughout the contents of a single host cell and takes place very rapidly since it is seen only in a few cells in any one section and is not always present. At a slightly later stage the protoplasm of the plasmodium becomes aggregated into dense spherical masses which later become surrounded by clear areas or walls (Fig. 1*e*–*g*). When such a mass is seen free from host tissue in gently squashed hand or freezing microtome sections of fresh material, the spherical bodies separate slightly from one another and single specimens on the periphery of the mass appear to give rise to minute particles

($<0{\cdot}5\mu$. dia.) which move away sluggishly leaving the collapsed vesicle. In sections of fixed material the contents of the cluster of spheres stain deeply with gentian violet or iron alum haemotoxylin. These deeply stained masses are at first regularly granular and finally break up unto small particles ($<0{\cdot}5\mu$. diam.) which are liberated (Fig. 1 *h–i*). Empty collapsed vesicles, comparable to those seen in fresh squashed material, can also be seen in the stained sections (Fig. 2). The small bodies liberated from a cluster of vesicles flow in a muci-

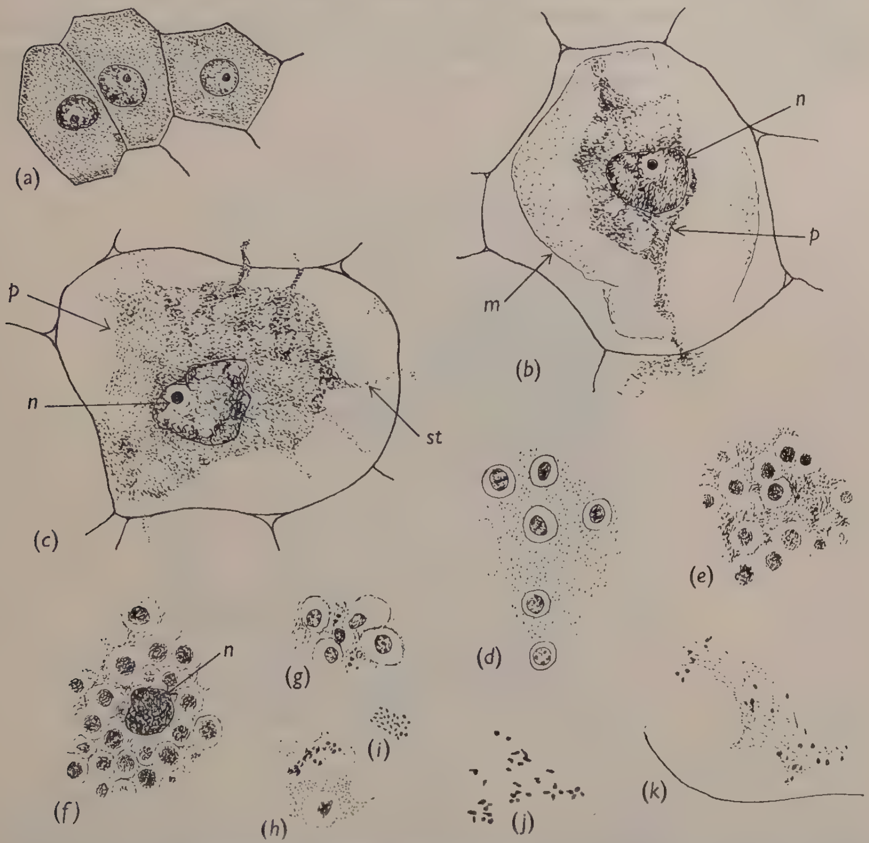

Fig. 1. *Hippophae rhamnoides*. Stages in development of endophyte. *a*, uninfected meristematic cells from apex of nodule. *b*, cell from zone behind apex (zone *P*, Pl. 1, fig. 1) showing young plasmodium (*p*) which has entered cell through a small pore in the wall and has surrounded the host nucleus (*n*). (*m*) remains of plasma membrane. *c*, cell from same zone, slightly more advanced stage of infection. Plasmodium (*p*) half-filling cell, host nucleus (*n*) showing erosion. Strands (*st*) extending to host cell walls and into adjacent cells. *d* to *f*, stages in the formation of sporangia (zone *S* of Pl. 1, fig. 1). *d*, shows dividing nuclei of endophyte. *e*, later stage in the formation of sporangia. *f*, deformed host nucleus (*n*), surrounded by mature sporangia. *g*, shows presence of definite 'bacteroid'-like particles or granules in sporangium. *h*, shows these escaping from sporangium. *i*, shows the escaped 'bacteroids'. *j*, shows larger bodies which may be the result of conjugation of the original granules. *k*, shows disintegrating remains of endophyte from zone *D* (Pl. 1, fig. 1), with a few bacteroids still present. (All drawings ×1200.)

laginous mass into neighbouring cells and intercellular spaces. Examples have been noted where the mucilaginous masses from different cells flow together and in these intermingled masses the granules are approximately twice the size of the newly liberated ones (Fig. 1*j*), suggesting a conjugation of granules derived from different clusters. At this stage the walls of the host cells show a change in nature, indicated by an alteration in staining properties, and are often partly disintegrated and absorbed.

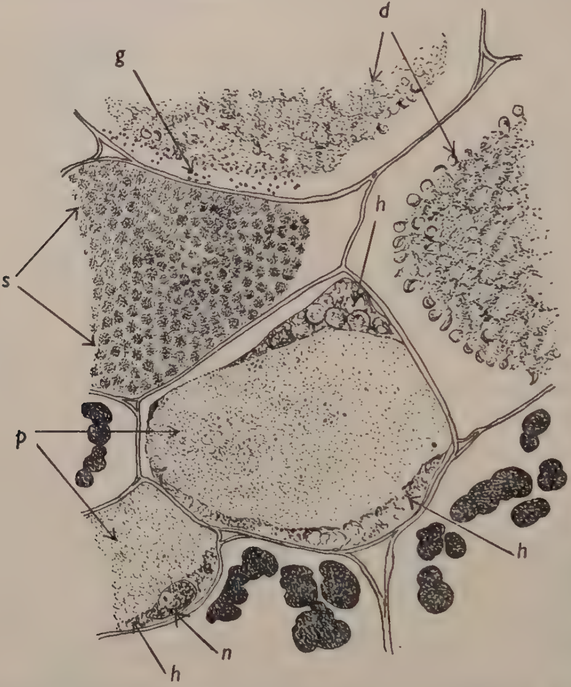

Fig. 2. ***Elaeagnus macrophylla.*** **Stages in development of endophyte (from same slide as Pl. 1, fig. 3). Plasmodium (*p*) nearly filling host cell and beginning to form sporangia, (*h*) remains of host cytoplasm, (*n*) host nucleus, (*s*) young sporangia, (*d*) disintegrating remains of plasmodia and sporangia with some bacteroid-like granules (*g*) escaped from sporangia. The nodule from which this section was cut was not actively growing (see p. 373) and the zonation was thus less distinct than in Pl. 1, fig. 2. Dark amorphous masses in some cells are host contents. × 550.**

The third zone of the infected part of the nodule lies at the base and its extent depends on the age of the nodule. Here the host cells are almost empty and the walls are much broken and disintegrated. In the cells nearest the active zone of infection disintegrating remains of collapsed vesicles (Fig. 1*k*) and protoplasmic strands may be seen, but further towards the base of the nodule few traces of the endophyte remain. It is not clear what becomes of the parasite at this stage. The most likely explanation is that the bulk of the protoplasm of the organism is used up in the formation of the small particles and that these escape into the soil by some means. These minute bodies are readily washed out of microtome sections during staining once they have escaped from the host cell in which they were formed, and they are too small to be identified with any certainty in fresh unstained material. In some of the preparations of *Hippophae*, however, these granules were observed in cracks in the cork coat at the base of the nodules whence they might be expected to pass out into the soil. Such cracks frequently arise as a result of renewed growth of the nodule following a return of favourable soil conditions, and it may well be that the granules normally escape in this way. In *Elaeagnus* the cells of each zone are not so uniformly infected as in *Hippophae*, and neighbouring cells which have hitherto escaped infection are invaded by the granules and a 'plasmodium' subsequently develops. It is also possible that the protoplasm of the plasmodium stage may flow out into the soil as such masses of protoplasm have occasionally been seen in cracks in the cork coat of the nodule. There is no means of determining whether such a mass is entering or escaping from the nodule. A similar migrating plasmodium has been figured by Schaede (1933) in a root-nodule of *Alnus japonica*, and is interpreted by him as showing penetration of the endophyte into the nodule, despite the fact that the nodule is already infected.

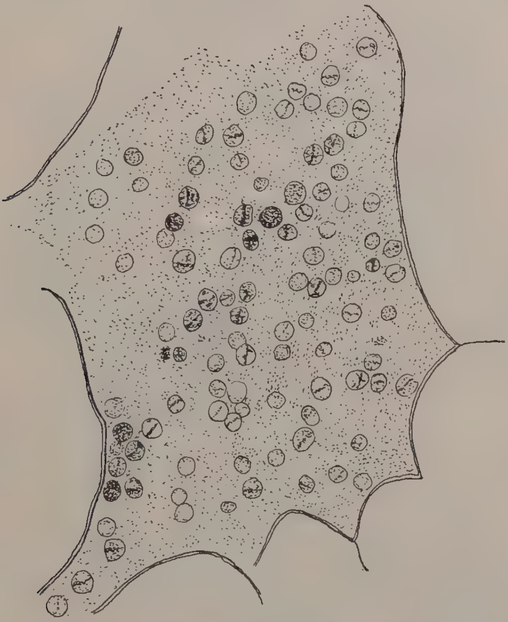

Fig. 3. ***Elaeagnus angustifolia*.** **Nuclear divisions in plasmodium (from same slide as Pl. I, fig. 4). Fully developed plasmodium of endophyte showing nuclear divisions (probably meiotic). × 1000.**

Whatever the method by which the endophyte escapes from mature nodules into the soil it is clear that young lateral roots become infected from the soil as they emerge from the surface of the parent root. There is a short period when the region just behind the root cap is quite unprotected. A cell completely filled with the invader has been seen in this position when there was no trace of the endophyte in either the parent root or the extreme tip of the rootlet. The invading organism probably obtains entry in the form of a free-moving particle from the soil.

Alnus glutinosa

The endophyte in root-nodules of *Alnus* is again confined to the cortex behind the growing point, but it occurs in tangentially-arranged plates of cells separated by normal ones containing large compound starch grains and others containing a dark amorphous substance (Pl. 2, fig. 1). The latter resemble the tannin cells characteristic of tree roots infected with ectotrophic mycorrhiza-forming fungi, and their presence suggests a host reaction to the presence of the invader whose migrations are thus restricted.

The morphology and life history of the endophyte are essentially similar to those of the endophytes of *Elaeagnus* and *Hippophae*. The vesicles are larger (6μ. diam.) and a bigger proportion of the protoplasm is used up in their formation so that instead of being embedded in a network of protoplasm they are attached to fine strands, giving them the appearance of a bunch of toy balloons (Fig. 4; Pl. 2, fig. 1). This is particularly clear in expressed fresh material (Fig. 5). The connecting strands of protoplasm linking the bunches of vesicles in adjacent cells are more substantial than those of the endophytes of *Elaeagnus* and *Hippophae*, but still have no definite cell wall and are too irregular in size and shape to be interpreted as hyphae.

The granules are more readily liberated from the vesicles in fresh material, when they tend to form a dense 'halo' round the expressed endophyte, than in the other two species. They are also seen more frequently in stained preparations of alder nodules (Pl. 2, fig. 2) than in those of *Elaeagnus* and *Hippophae*, but this may be due to greater resistance of the host walls to attack so that escape and migration of the granules are more difficult. Granules are frequently seen in the intercellular spaces, however, and the doubling of their size, presumably as a result of conjugation, has been most clearly seen in this species (Fig. 6).

Myrica gale

The endophyte in root-nodules of *M. gale* shows some important differences from those of the hosts already considered. It occurs throughout the cortex and shows a somewhat similar zoning of the infected area, but the parasite has a particularly striking effect upon the cells of the host at an early stage of infection. Invaded cells are much enlarged, the nucleus is rapidly absorbed, and the walls are altered so that they give a yellow reaction with aniline chloride which suggests lignification, as pointed out by Schaede (1939). The invading organism has difficulty in penetrating these walls. Passage of the plasmodium from one cell to another takes place by way of small pores in the host wall which have presumably been made by the invader (Fig. 7*b*). Frequently funnels of host-wall material extend into the cell from the pore (Fig. 7*c–e*;, Pl. 2, fig. 3) and are no doubt a host reaction to penetration, reminiscent of that seen in response to the penetration of clover by *Rhizobium leguminosarum* (Thornton, 1949) and of various plants by such fungi as *Erysiphe polygoni*. The result is that at each cross-wall the plasmodium is restricted to a narrow channel and oozes through the pore in a thick stream

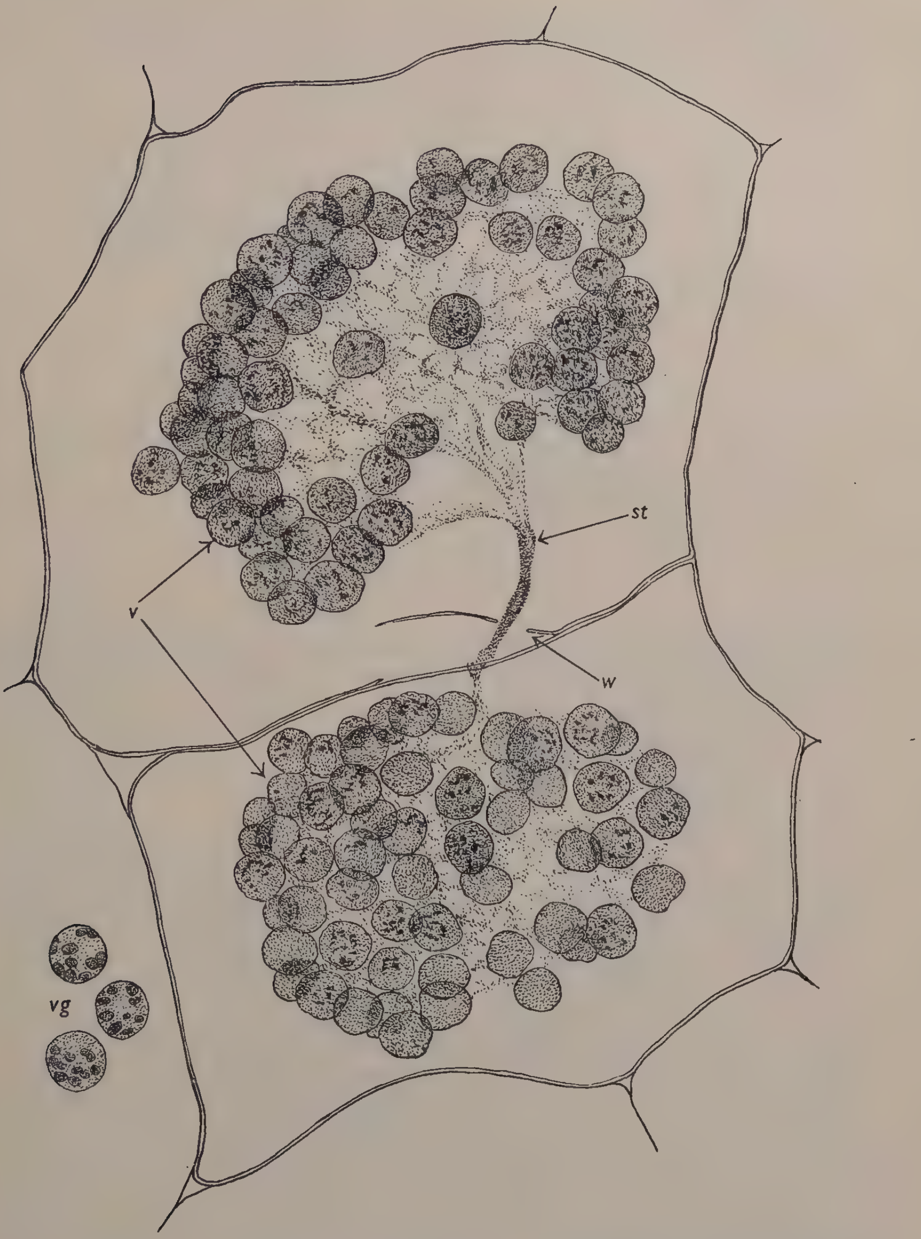

Fig. 4. *Alnus glutinosa.* Vesicular stage of endophyte. Vesicles (sporangia (*v*)) of endophyte in longitudinal sections of cells of root-nodule. Note connecting strands of protoplasm (*st*), broken cell wall of host (*w*) at point where connecting strands pass through. (*vg*) = slightly older vesicles showing formation of granules. × 1050.

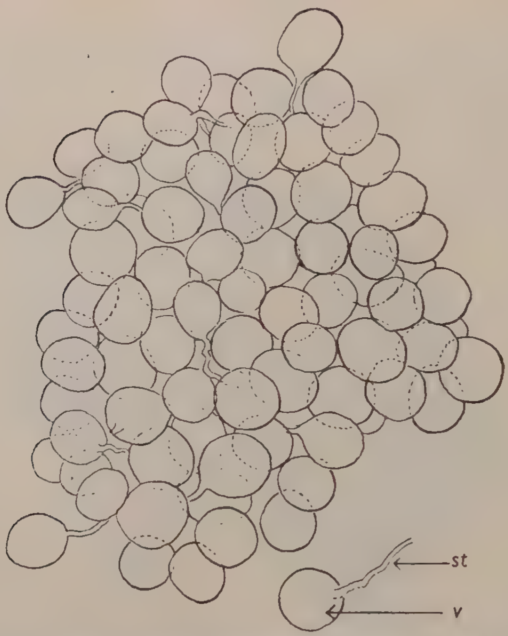

Fig. 5. *Alnus glutinosa*. Fresh material, squash preparation. Mass of vesicles (sporangia) of endophyte freed from host cell by gentle squashing. (*v*) = single vesicle; note attached protoplasmic strand (*st*). × 1050.

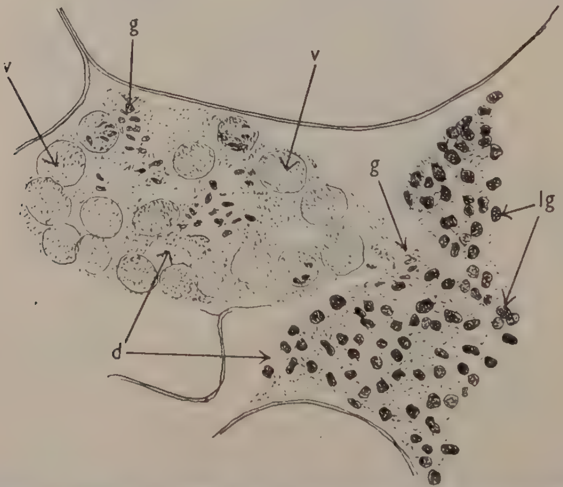

Fig. 6. *Alnus glutinosa*. Migrating granules of endophyte. Empty vesicles (*v*) of endophyte. 'Bacteroid'-like granules (*g*) which have escaped from the vesicles, and larger granules (*lg*) which are probably the result of conjugation of the smaller ones. (*d*) disintegrating remains of reticulate plasmodium. × 1000.

which continues across the cell to the opposite wall and which may often be traced through several cells in one section (Fig. 7 *a–c*; Pl. 2, fig. 3). Elongated deeply staining granules are sometimes seen oriented in a direction parallel to the direction of flow of the protoplasm, as would be expected. The plasmodium fans out from this central strand to fill the periphery of the host cell with an amorphous or somewhat reticulate mass of protoplasm.

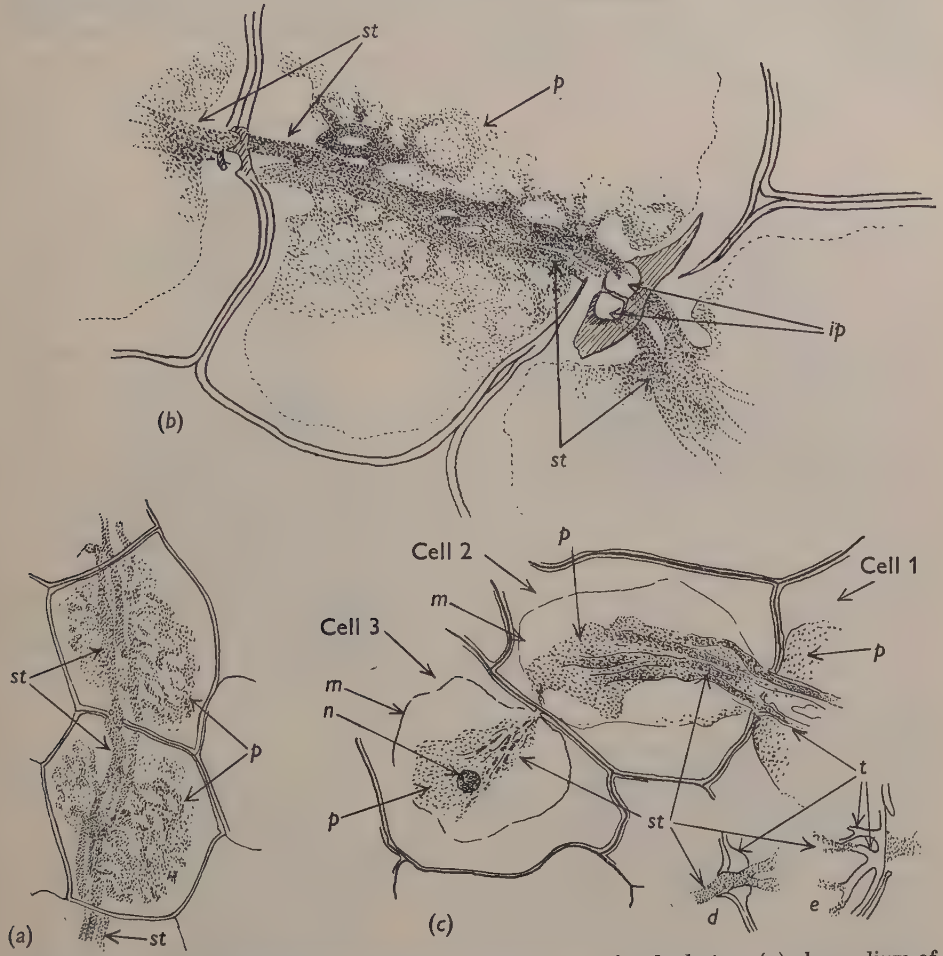

Fig. 7. *Myrica gale*. Plasmodia and protoplasmic strands of endophyte. (*a*) plasmodium of endophyte. *st*, protoplasmic strands or 'lines of flow' which pass through cell walls of host and fan out to fill cell with a reticulate mass of protoplasm (plasmodium, *p*). × 900. (*b*) similar stage showing infection pores (*ip*) in host cell wall. Wall is disintegrating. × 1500. (*c*) cells shown in Pl. 2, fig. 3. Note host nucleus (*n*) and plasma membrane (*m*) in cell 3, 'lines of flow' or protoplasmic strands of endophyte (*st*) and tubular outgrowths (*t*) from host cell wall in response to presence of parasite. × 900. *d* and *e* show similar lignified outgrowths of cell wall of host which confine the plasmodium in its passage from cell to cell. × 900.

Instead of the spherical vesicles seen in the previously described organisms, club-shaped structures develop at the periphery of the plasmodium of the

Myrica endophyte (Fig. 8*a*; Pl. 2, fig. 4). These stain deeply and uniformly with gentian violet, but their contents are seen to be granular in preparations stained with iron alum haemotoxylin (Fig. 8*b*). There is no distinct membrane surrounding them as round the vesicles of the other parasites considered here, and the club-shaped bodies break up into packets of small particles resembling those formed within the vesicles of the other organisms (Fig. 8*c*). These club-shaped bodies must break up rapidly after formation as they are seldom seen. In most preparations only the plasmodial and the particle stages are present (Fig. 8*c*, *d*; Pl. 2, fig. 5). In squash preparations made from thin sections of fresh material, granules have frequently been seen to detach themselves from

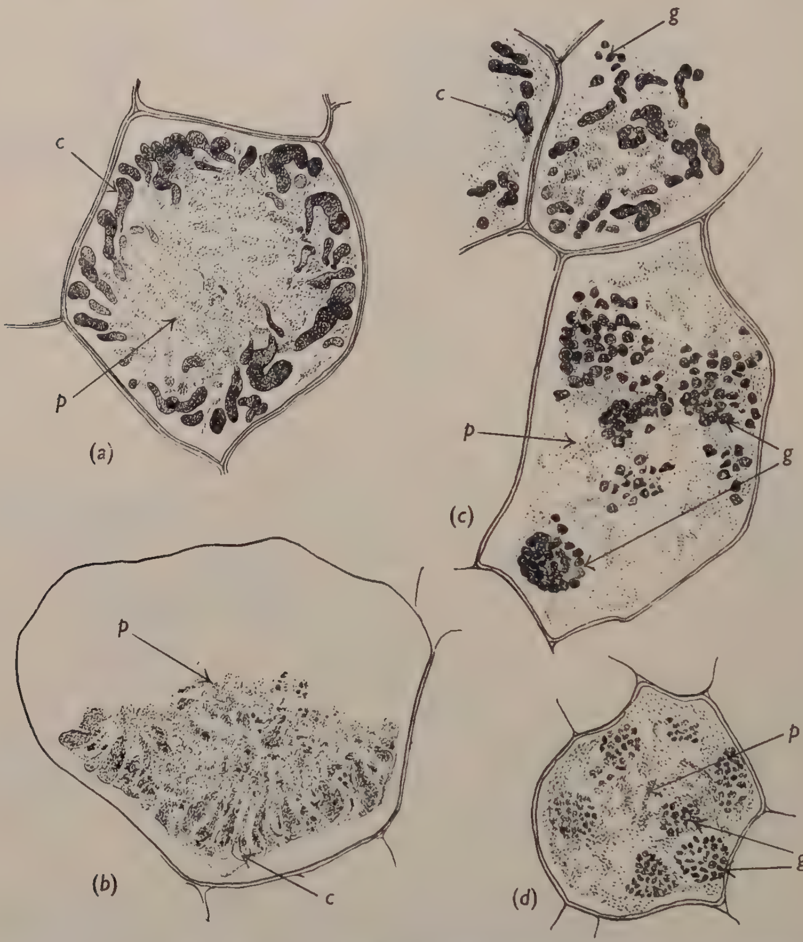

Fig. 8. *Myrica gale*. Production of granules. (*a*) plasmodium (*p*) of endophyte, forming club-shaped bodies (*c*) at periphery. Stained gentian violet and light green. × 1050. (*b*) similar cell (from same nodule) stained iron alum haematoxylin, showing granular contents of clubs. × 1050. (*c*) cells from same preparation as (*a*) showing breaking up of club-shaped bodies to give 'bacteroid'-like granules (*g*). × 1050. (*d*) slightly later stage showing typical groups of granules. × 600.

extruded masses of endophyte in the 'particle' stage and to move away slowly with a dancing movement. These particles are regular in size ($0{\cdot}5\mu$. long) and are tadpole-shaped with the posterior end pointed. In sections these masses of particles are seen to be still enclosed within the plasma membrane of the host, even though all other host cell contents have been consumed, so that it is difficult to see how the separate bodies migrate unless they do so by way of the pores previously made by the protoplasmic strands which presumably pierced both membrane and cell wall.

DISCUSSION

Relation between the endophytes of Elaeagnus, Hippophae, Alnus *and* Myrica. The morphology and general life history of these endophytes is similar. All show a plasmodial stage and all finally produce minute migratory particles. The endophytes of *Elaeagnus* and *Hippophae* are morphologically indistinguishable and almost certainly belong to the same species. In experiments which are still in progress, and which are to be repeated, cross-infection of these two hosts took place. The endophyte of *Alnus* is closely related but differs in the greater size of the vesicles and in the almost complete utilization of the protoplasm of the plasmodium during their formation. The endophyte of *Myrica* shows greater differences, notably the formation of the particles by the breaking up of club-shaped bodies, and may well be placed in a separate genus.

Nature of the endophyte. The endophytes of the root-nodules of species of *Elaeagnus, Hippophae, Alnus, Myrica* and *Ceanothus* have been variously interpreted as filamentous fungi (Hyphomycetes or Phycomycetes), bacteria, actinomycetes or members of the Plasmodiophorales.

The first record is that of Woronin (1866), who described an organism in the roots of *Alnus glutinosa* which he called *Schinzia alni* and interpreted as a filamentous fungus belonging to the Hyphomycetes. Moeller (1885) placed it in the Plasmodiophorales as *Plasmodiophora alni* (Woron) Moeller, and figured the migrating plasmodia. Schroeter (1889, 1897) agreed with Moeller and placed the endophyte of *Elaeagnus angustifolia* also in the genus *Plasmodiophora* (as *P. elaeagni*). Brunchorst (1886), however, could find no plasmodial stages in root-nodules of *Alnus* spp., *Elaeagnus* spp., *Hippophae rhamnoides* and *Myrica gale* and describes septate hyphae with terminal sporangia. He called these organisms *Frankia subtilis* and, notwithstanding their septate hyphae and small size, considered that they were related to the Mucorales or Saprolegniales. The views of Woronin and Brunchorst were then supported by Moeller (1890), who withdrew his earlier interpretation of the alder organism as a species of *Plasmodiophora*, and by Frank (1891) who considered *Frankia subtilis* to be a mycorrhizal fungus, although he pointed out the possibility of its being a thread-forming bacterium allied to *Leptothrix*. Atkinson (1892) demonstrated the resemblance of the organism inhabiting root-nodules of *Ceanothus americanus* to both *Plasmodiophora brassicae* and the alder organism described by Woronin as *Schinzia alni* and renamed *Frankia subtilis* by Brunchorst (1886). Hiltner (1896) claimed that the root-nodules of *Alnus* and *Elaeagnus* contained bacteria which he suggested were able to fix nitrogen. Chodat (1904) also inclined to the view that the endophytes were bacteria but with some myxomycete and actinomycete characters. That the question was at that time highly controversial is shown by Shibata's (1902) description of the *Myrica* endophyte as an actinomycete showing threads which broke up into bacteroid-like particles, by Björkenheim's (1904) claim to have seen fungal hyphae in the nodules, by Keissler's (1907) reference to the alder organism as *Plasmodiophora alni* and by Jaap's (1907) report of the occurrence of *P. elaeagni* in Switzerland. In a cytological investigation of the Plasmodiophoraceae Maire & Tison (1909) excluded *P. alni* and *P. elaeagni* from this group and agreed with Shibata. They described tenuous branched filaments, containing chromatin granules but with no membrane and no definite nucleus, and developing terminal vesicles which segmented into a number of small irregular bodies. They renamed the *Alnus* and *Elaeagnus* endophytes *Frankiella alni* and *F. elaeagni*, and placed them in the Schizomycetes. Peklo (1910) supported Shibata as to the actinomycetous nature of the *Myrica* organism. Bottomley (1912, 1915) and Spratt (1912), however, claimed that the organisms in the root-nodules of *Alnus, Elaeagnus, Myrica* and *Ceanothus* were identical with the nitrogen-fixing bacteria in root-nodules of the Leguminosae. Both Bottomley and Spratt supported this view by claims of successful cross-inoculation experiments, but the reference which they give (Bottomley, 1907) does not mention such experiments.

Ziegenspeck (1929) and Borm (1931) sought to reconcile the opposing views of the various investigators by interpreting the 'plasmodial' stages as disintegration stages resulting from the development of a bacteriophage which was supposed to attack the nodule-forming bacteria. Schaede (1933, 1939) and Roberg (1934, 1938) have more recently supported Shibata's claim that these organisms are actinomycetes, but Keissler & Lohwag (1937) have once more referred the alder organism to *Plasmodiophora*, while Sydow (1924) and Yendo & Takase (1932) describe the *Elaeagnus* endophyte as *Plasmodiophora elaeagni* and *Tetramyxa elaeagni* respectively. Plotho (1941) claims to have isolated the alder organism and calls it *Actinomyces alni*. As an aid to clearness these conflicting views are classified in Table 1.

Claims that these organisms are filamentous fungi, either Hyphomycetes or Phycomycetes, are untenable since they are devoid of cell walls, and the protoplasmic strands are of variable width and of too small a size to be interpreted as fungal hyphae. The fact that a second endophyte, an arbuscular-vesicular type of endotrophic mycorrhizal fungus, is frequently seen in roots of *Elaeagnus angustifolia* suggests that some earlier writers may have confused the two organisms. The fungus, however, is present in the cortex of otherwise normal roots and is never seen in the nodules.

The theory that the protoplasmic strands seen by many observers are the hyphae or threads of an actinomycete is more plausible. In fixed material, particularly of *Myrica gale*, the protoplasmic strands have something of the appearance of fine hyphae, but examination of material fixed with modern fixatives shows that there are no cross-walls and no obvious containing walls, and that the width and branching of the strands are very variable. In fresh material these strands are even less like hyphae. The striated appearance

Table 1. *Interpretation by various investigators of the nature of the endophytes of the root-nodules of non-leguminous plants*

Interpretation of organism	Host*	Reference
Bacterium	*A, E*	Hiltner (1896)
	A, H	Chodat (1904)
	C, M	Bottomley (1907, 1912, 1915)
	E	Spratt (1912)
Bacterium (attacked by bacteriophage)	*A, H*	Ziegenspeck (1929)
	A, H	Borm (1931)
Actinomycete	*M*	Shibata (1902)
	A, E	Maire & Tison, (1909)
	M	Peklo (1910)
	M	Youngken (1919)
	A, H, M	Schaede (1933, 1939)
	A, E, H	Roberg (1934, 1938)
	A	Plotho (1941)
Filamentous fungus	*A*	Woronin (1866)
	A	Moeller (1890)
	A, E, H, M	Brunchorst (1886)
	A, E	Frank (1887, 1891)
	M	Björkenheim (1904)
	A, E	Zach (1908)
Member of Plasmodiophorales	*A*	Moeller (1885)
	A, E	Schroeter (1889, 1897)
	C	Atkinson (1892)
	E	Jaap (1907)
	A	Keissler (1907)
	E	Sydow (1924)
	E	Yendo & Takase (1932)
	A, E, H, M	Hawker & Fraymouth (present paper)

* *A = Alnus, C = Ceanothus, E = Elaeagnus, H = Hippophae, M = Myrica*

sometimes seen (Fig. 7) is due to the orientation of elongated particles along the direction of protoplasmic streaming across a cell from one pore to another. The protoplasm which fans out from the strands into the periphery of the cell or into a newly invaded cell is amorphous or indefinitely reticulate and thus bears no resemblance to the threads of an actinomycete. Attempts to interpret this amorphous mass as due to the disintegrating effect of a bacteriophage are unfortunate, as the 'plasmodial' stage actually precedes the protoplasmic strand and 'bacteroid' stages in the infection of any particular cell. The club-shaped bodies seen occasionally in the endophyte of *M. gale* bear a superficial resemblance to those produced by certain actinomycetes. The production of groups of bacteroid-like particles from these bodies differs from the fission of the hyphae of true actinomycetes and is not paralleled in the development of the terminal 'clubs' of the latter. Moreover, the spherical vesicles of the endophytes of *Alnus*, *Elaeagnus* and *Hippophae* are unlike any structures seen in that group. Claims based on the reaction to the Gram stain and other stains are of little value since these reactions vary with the stage of development of the parasite.

The claim made by Bottomley (1912, 1915) and Spratt (1912) that the endophytes of root-nodules of non-leguminous plants are identical with *Rhizobium* *leguminosarum* is untenable in view of the prevalence of plasmodial and sporangial stages. The observations of these workers must have been based solely on cells in which the endophyte was represented by the small bacteroid-like particles formed from the spherical or club-shaped sporangia. At this stage many cells may contain these particles, which bear a superficial resemblance to *R. leguminosarum* but do not show the typical T and Y forms or the characteristic banding of the bacteroids of this organism. The fact that neither we ourselves nor Miss S. Mount (unpublished) have been able to cultivate these organisms by methods which sufficed for the isolation of the clover bacterium is additional evidence that they are not identical or even similar. Bottomley's claim (Spratt, 1912) that successful cross-inoculations were made between leguminous and non-leguminous hosts may have been based on faulty experimental technique. The present writers have reason to believe that the *Elaeagnus* and alder organisms are widely distributed in ordinary garden soil so that contamination might easily have taken place.

There remains the hypothesis that these organisms are members of, or at least closely related to, the Plasmodiophorales. The present observations are consistent with this view. The plasmodial stages are strikingly similar to those of *Plasmodiophora brassicae* with the exception of the greater tendency to form protoplasmic strands along the lines of flow. The development of these strands may be correlated with the degree of resistance of the host walls so that when resistance is considerable the area of penetration is reduced to a small pore and the flow of the plasmodium thus canalized into narrow streams. Thus in *Myrica* the endophyte shows the most definite development of protoplasmic strands or channels of flow and the walls of the host cell show the most definite reaction and rapidly become lignified in response to the presence of the parasite. In *Alnus*, which shows less distinct strands, resistance takes the form of the production of tannin cells and the walls of the invaded cells show only slight alteration and readily break down. In *Elaeagnus* and *Hippophae*, where the walls show little if any reaction to invasion and readily disintegrate, the invader shows very little development of protoplasmic strands and is frequently seen in the intercellular spaces. In all these, invasion is limited to the nodules which may, however, attain considerable size and complex branched structure. In *Plasmodiophora brassicae*, where resistance is slight and large areas of the root become involved, the cell walls offer little or no resistance to penetration, and consequently the plasmodium shows no development of protoplasmic strands corresponding to channels of flow but moves as an amoeboid mass.

The production of definite spherical sporangia by the endophytes of *Elaeagnus*, *Hippophae* and *Alnus* and of club-shaped ones by that of *Myrica* is no obstacle to the inclusion of these organisms in the Plasmodiophorales since modern work (Cook & Schwartz, 1929, 1930; Cook, 1933; Karling, 1942) has suggested the existence of a sporangial stage in *Plasmodiophora brassicae* and in some other members of the group.

The sluggish movements of the released particles suggest that these may be myxamoebae rather than zoospores, but the presence or absence of flagella could not be determined with certainty.

The chromatin granules present in the plasmodium are very small and no mitotic nuclear divisions were observed. Where nuclear divisions were observed (in *Elaeagnus* (Fig. 3; Pl. 1, fig. 4) and *Hippophae*) these were probably meiotic since they preceded the development of the sporangia. If they were reduction divisions, one would not expect them to be of the peculiar cruciform type described by some observers (Horne, 1930; Cook, 1928, 1933; Webb, 1935) as typical of mitotic divisions in some members of the Plasmodiophorales. No such cruciform divisions were seen in any of the nodule organisms here described.

Thus the morphology and life histories of the endophytes of *Elaeagnus*, *Hippophae*, *Alnus* and *Myrica* suggest that they are best placed in the Plasmodiophorales. The old names *Plasmodiophora elaeagni* and *P. alni* have much to recommend them, while the *Myrica* organism might best be placed in a separate genus based on the club-shaped sporangia and the very marked protoplasmic strands. This organism is in many ways an approach to the actinomycetes and indicates a possible means of the evolution of coenocytic hyphae by the development of protoplasmic strands along lines of flow in a plasmodium and the subsequent condensation of the outer layers of these to form a membrane or wall. All these organisms show characters, such as the formation of definite sporangia, which approach the true fungi and they thus afford evidence in support of the view that the Plasmodiophorales are indeed primitive fungi.

The physiological nature of the host-parasite relationship is still a matter of doubt. Claims have been made that these organisms are able to fix nitrogen, but some observers have found no evidence of fixation. In our preliminary experiments and in those of Miss S. Mount (unpublished) seedlings of *Hippophae rhamnoides* grew to a height of 6 in. in sterilized sand without additional nitrates and were in no way inferior to inoculated seedlings with well-developed nodules, while seedlings of *Alnus glutinosa* in garden soil were equally vigorous whether they bore nodules or not. These experiments are being continued and extended. Naturally and artificially inoculated seedlings of these plants do not develop nodules until they have reached a considerable size, whereas inoculated clover seedlings develop them soon after germination. This suggests that the non-leguminous hosts are less dependent on, or even independent of, the nodule organism. The failure to isolate the endophytes by methods sufficing for the cultivation of *Rhizobium* suggests that the relationship may be one of obligate parasitism as with other species of *Plasmodiophora*. In these examples, however, the parasite is more or less controlled by the resistance of the host. In this connexion it is of interest that material of species of *Ceanothus* obtained by the writers and by Bottomley (1915) from various sources in England was devoid of root infection, but the plants were well developed, thus indicating that they were independent of the nodule-forming endophyte found by other workers in American material.

Thanks are due to the Director and Curator of the Royal Botanic Gardens, Kew, for seeds of various species and root material of *Ceanothus* spp., to Miss S. Mount for permitting us to use her slides of *Hippophae rhamnoides* for comparison with ours

and for undertaking preliminary cultural studies, to Prof. Hewer of the Department of Pathology, University of Bristol, for allowing the use of his apparatus for taking photomicrographs and to Mr Rodgers for taking the photographs.

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EXPLANATION OF PLATES

PLATE 1. *Hippophae rhamnoides* and *Elaeagnus* spp.

Fig. 1. Median longitudinal section of root-nodule of *Hippophae rhamnoides*. *G*, growing point; *P*, zone in which host cells contain young plasmodium; *S*, zone in which endophyte is in the form of spherical sporangia; *D*, zone in which endophyte is disintegrating. ×18.

Fig. 2. Median longitudinal section of root-nodule of *Elaeagnus macrophylla*. Lettering as in Pl. 1, fig. 1. ×25.

Fig. 3. *E. macrophylla* cells from edge of sporangial zone. *p*, plasmodium of endophyte; *n*, host nucleus; *h*, remains of host cytoplasm; *s*, sporangia of endophyte. ×400.

Fig. 4. *E. angustifolium*. Fully developed plasmodium of endophyte showing nuclear divisions (probably meiotic). ×800.

PLATE 2. *Alnus glutinosa* and *Myrica gale*.

Fig. 1. Infected cells in longitudinal section of root-nodule of *Alnus glutinosa*. Endophyte in vesicular or sporangial stage. *v*, vesicles; *st*, protoplasmic strands connecting endophyte in adjacent cells; *t*, tannin (?) cells of host; *sg*, starch grains in host cell. ×400.

Fig. 2. 'Bacteroid' stage of endophyte in *A. glutinosa*. These bacteroid-like granules (*g*) are formed in the vesicles or sporangia, disintegrating remains of plasmodium with empty vesicles (see fig. 1). ×400.

Fig. 3. Plasmodium of endophyte of *Myrica gale* advancing towards tip of nodule. 1, 2 and 3 are host cells in various stages of infection. Cell 1 is filled with endophyte, cell 2 is not completely filled and the plasma membrane (*m*) of the host is still visible, cell 3 has only just been entered. *st*, protoplasmic strands of endophyte; *p*, plasmodium, fanning out from these. Note tubular thickened mass of host cell wall (*w*) at point where parasite has entered cell 2 from cell 1. ×500.

Fig. 4. Club-shaped bodies (*c*) of the endophyte of *M. gale* forming at periphery of plasmodium (*p*) (stained gentian violet). 'Bacteroid' stage (*g*), at lower left hand corner. ×200.

Fig. 5. Groups of 'bacteroids' (*g*) formed in cells of nodule of *M. gale*. ×200.

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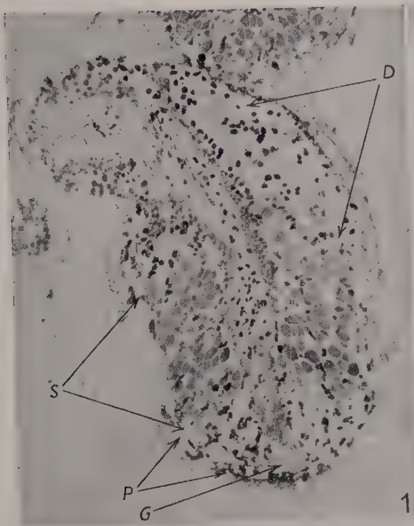
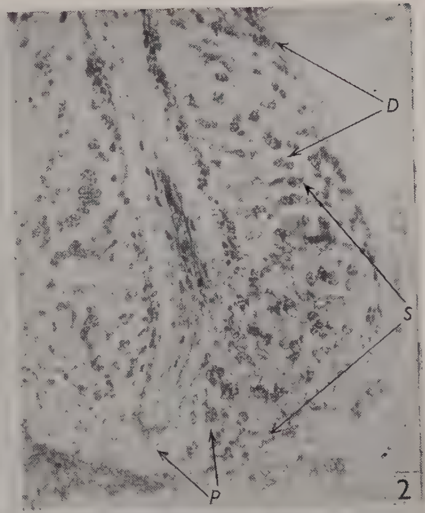
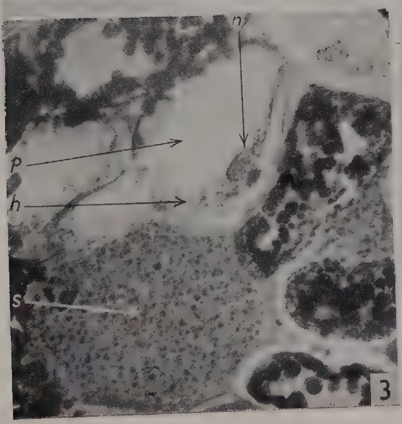
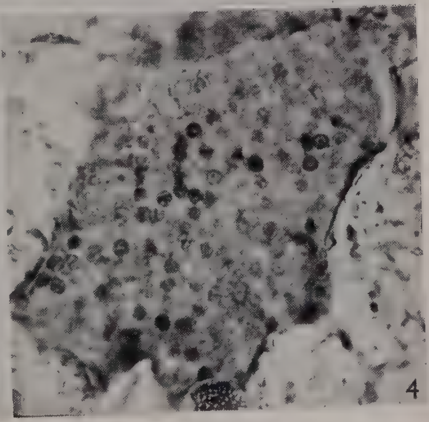
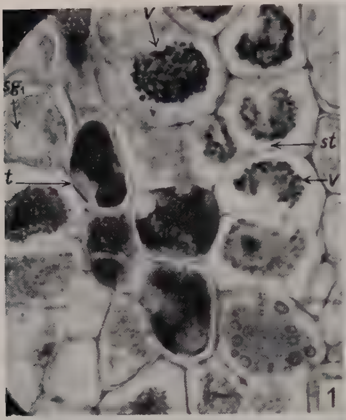


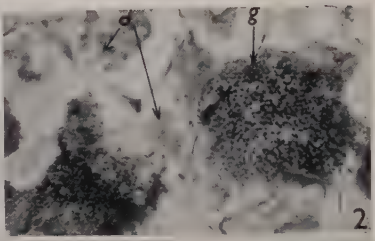


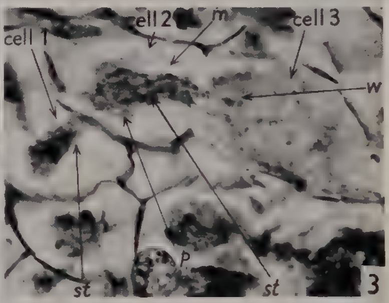


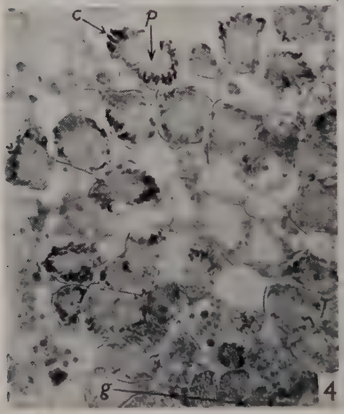


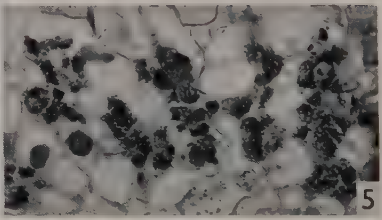
Lilian E. Hawker & Joan Fraymouth—Plasmodiophorales in root-nodules. Plate 1











Lilian E. Hawker & Joan Fraymouth—Plasmodiophorales in root nodules. Plate 2

Pearce, T. W. & Powell, E. O. (1951). *J. gen. Microbiol.* 5, 387–390.

A Selective Medium for *Bacillus anthracis*

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SUMMARY: A nutrient medium containing lysozyme and haematin is described which permits free growth of *B. anthracis* while suppressing more than 95 % of other *Bacillus* spp. in soil.

The work of Chu (1950; see also McGaughey & Chu, 1948) provides criteria which greatly facilitate the differentiation of *B. anthracis* from other spore-bearing aerobes. When, however, *B. anthracis* is to be searched for in samples of soil, plant or animal material, the problem is complicated by the presence, often in very large numbers, of organisms having many similar characters. Any simple means of even partly segregating the *B. anthracis* is obviously of value. We have devised an inhibitory medium which allows *B. anthracis* to grow freely while suppressing the greater proportion of normal soil organisms of the same genus (and in fact of most other genera). The medium thus selects a small fraction in which *B. anthracis*, if present, will be found, and subsequent identification is correspondingly less laborious.

We made use of two sets of observations: (i) van Heyningen (1948) showed that the spore-bearing aerobes could be divided into species which would, and those which would not, grow on nutrient agar containing 50 μg. haematin/ml. *B. anthracis* was not inhibited; we have confirmed this observation with four strains in our possession. We have also noted that there are commonly present in soil strains of *B. mycoides* which grow freely on such a medium. (ii) D. Herbert (private communication) has found that the genus *Bacillus* may be divided into two groups of species on the basis of resistance to lysis by lysozyme. He found that six species (66 strains) were non-resistant, five (58 strains) were resistant (among them *B. anthracis*) and four (57 strains) differed in resistance from strain to strain. Our experiments indicate that some species whose cells are non-resistant to simple buffered solutions of lysozyme will nevertheless grow in nutrient media containing enough lysozyme to destroy them in the absence of nutrients. Two other factors favour the growth of *B. anthracis*. First, many soil organisms grow poorly or not at all at 37° or higher; secondly, the effectiveness of inhibitors usually increases with temperature. *B. anthracis* grows well up to 40°.

PREPARATION AND USE OF THE MEDIUM

The medium consists of 1 vol. of solution (*a*): 400 μg. haemin/ml. in 0·01 N-NaOH (autoclaved at 5 lb./sq.in. for 30 min.) and 1 vol. of solution (*b*): 600 μg. lysozyme/ml. in 0·01 N acetic acid (heated to 50–60° for 15 min. before use) added to 8 vol. of peptone agar (Evans's peptone 2 %, NaCl 0·5 %, agar 2 %, pH 7·4) which has been cooled to 60° after melting. Plates are poured immediately, since remelting destroys much lysozyme. The pH of the peptone agar is not appreciably changed when solutions *a* and *b* are added.

Inocula are prepared by grinding the sample of soil or other material with a little water and diluting appropriately—a final dilution of 10^3–10^4 ml./g. was found satisfactory for the soils we investigated. The suspension is heated to 60° for 30 min. before inoculation; this encourages the germination of spores (Powell, 1950) as well as killing most non-sporing species.

Incubation may be carried out at 37·5° or 40° for 16–20 hr. The selectivity of the medium is better at the higher temperature, the almost complete absence of *B. mycoides* being often a notable advantage. There is then, however, some loss of *B. anthracis*; up to 20 % of the spores fail to produce visible colonies within 20 hr. At both temperatures the growth rate of *B. anthracis* is obviously but in no way seriously decreased. Microscopic examination of the inoculum shows that vegetative cells are produced after about 1½ hr. as usual, but the colonies are ultimately half to two-thirds the diameter of controls, and more convex, while the loops of growth do not extend so far into the outlying medium.

Of the organisms which do grow on the selective medium, a fair proportion can be disregarded at once on grounds of their colonial appearance, e.g. *B. mycoides* and *B. circulans* are readily recognized.

GROWTH TESTS

Tests were made of the ability of a few species to grow on the medium from spore inocula. The following results refer to the presence or absence of macroscopic colonies after 16–20 hr. incubation. Four strains of *B. anthracis*, one of *B. mycoides* and three of *B. circulans* grew up to 40°; four strains of *B. subtilis*, one of *B. mycoides* and two of *B. megatherium* would not grow above 35°; and one strain each of *B. cereus*, *B. licheniformis* and *B. mesentericus* would not grow above 30°. These temperatures do not represent absolute limits, but only indicate the range over which tests were made.

Growth tests on total soil organisms give a better idea of the average performance of the medium. It is more important that it should inhibit a few of the commoner species than many of the rarer. We tested soils from three localities; chalk downland top soil and cultivated alluvial soil from Wiltshire, and soil from a very old pasture in Berkshire. There were no obviously significant differences in the purely numerical efficiency of the medium as between the different soils. There follows a record of two experiments.

Soil (1 g.) was ground with water (30 ml.), the suspension diluted to 1 l. and a portion of it heated to 60° for 30 min. Three sets of plates were prepared using: medium A, peptone agar + 50 μg. haematin + 60 μg. lysozyme/ml.; medium B, peptone agar + 25 μg. haematin + 60 μg. lysozyme/ml.; control medium, peptone agar alone.

Six plates of each medium were given equal inocula of soil suspension (5 drops, each 0·03 ml.) from a calibrated pipette. Three of each set were incubated overnight at 39–40°, three at 37–38°. The experiment was repeated exactly on another occasion with a sample of the same soil. The colony counts obtained are shown in Table 1 under Exps. 1 and 2. These figures in Table 1 are extremes; the statistical variation is great, especially since even a large inoculum contains only small numbers of some species. There is considerable real variation in both number and kind of organisms from one locality to another and from one soil level to another in the same locality, as well as a secular change. Consequently, there is little point in a detailed analysis of our results. As far as we can tell, variation of haematin concentration between 30 and 50 μg./ml., and of lysozyme between 40 and 80 μg./ml. makes no great difference to the overall efficiency of the medium. Parallel experiments in which different sources of agar and of lysozyme were used showed no obvious discrepancies. A grand total comprising the results of all experiments of the above type in which the ranges of inhibitor concentrations were those just quoted is therefore included in Table 1.

Table 1. *Growth of total soil* Bacilli *on the selective medium*

		37–38°		39–40°	
	Medium	Count	Count as percentage of control	Count	Count as percentage of control
Exp. 1	A	9	7	3	4
	B	14	11	5	7
	Control	130	—	76	—
Exp. 2	A	3	1	1	<1
	B	10	2	3	1
	Control	625	—	203	—
Grand total of 13 exps.	Selective:	174	4·1	58	2·1
	Control:	4240	—	2708	—

Of 58 colonies grown on the inhibitory medium at 39–40°, 8 (*c.* 15 %) were *B. mycoides*; of the 174 grown at 37–38°, 118 (*c.* 70 %) were of this species, many growing so poorly as to be scarcely visible, though occupying a large area.

We have also tried other inhibitor concentrations, with qualitatively similar results. We do not record them because satisfactory germination and growth of *B. anthracis* was not obtained when either the haematin concentration was above 50 μg./ml. or much in excess of the lysozyme concentration; i.e. at a fixed concentration of haematin, germination was better, the higher the lysozyme concentration. The quantities 40 μg. haematin + 60 μg. lysozyme/ml. are a good compromise.

Table 2 gives the results of two typical experiments illustrating the nearly unrestricted growth of *B. anthracis* on the selective medium. In both experiments, six plates of medium A and six of control medium were given equal inocula of suitably diluted suspensions of *B. anthracis* spores. Three of each set were incubated overnight at 37–38°, three at 39–40°. We think that the failure of some spores to germinate on the selective medium will not in most cases matter. More complete germination can be obtained by incubating for a longer period, say 40 hr., at a cost of slight decrease of effectiveness in suppressing other organisms.

Table 2. *Ability of* B. anthracis *to grow on the selective medium*

		37–38°		39–40°	
Strain	Medium	Count	Count as percentage of control	Count	Count as percentage of control
'M36'	A	1886	103	1727	81
	Control	1831	—	2143	—
'Weybridge'	A	891	90	749	79
	Control	994	—	946	—

USE OF EGG-YOLK MEDIUM

One of the simplest of Chu's criteria to apply is the positive egg-yolk reaction given by *B. anthracis*, *cereus* and *mycoides* (McGaughey & Chu, 1948). It seemed useful to obtain an impression of the frequency of organisms reacting with egg-yolk among those able to grow on the selective medium. Soil organisms which did so grow, therefore, were, unless obviously *B. mycoides*, transferred to egg-yolk peptone plates and incubated at 35°. The lower temperature itself effects a further selection; some of the *inocula* failed to grow. Of 143 colonies treated thus, 24 (*c*. 15 %) gave a positive egg-yolk reaction.

CONCLUSION

The medium described when incubated at 37–38° will suppress *c*. 95 % of those soil spores which will grow on ordinary peptone agar, leaving less than 5 % as contaminants of any *B. anthracis* which may be present. If a loss of about 20 % of *B. anthracis* can be tolerated, incubation may be at 39–40°, when the efficiency of the medium will be about doubled. The further use of the egg-yolk test decreases the number of contaminants possibly requiring more detailed examination to 1 % or less of their original number. Some of these can be disregarded, their colonial form being sufficient guarantee that they are not *B. anthracis*.

We are indebted to Mr L. H. Kent (Ministry of Supply) and Dr D. Herbert (National Institute for Medical Research) for preparations of lysozyme. Publication is by permission of the Chief Scientist, Ministry of Supply.

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The Adsorption of Cetyltrimethylammonium Bromide by Bacteria, its Action in Releasing Cellular Constituents and its Bactericidal Effects

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SUMMARY: The form of the uptake curve of cetyltrimethylammonium bromide (CTAB) by *Staphylococcus aureus* and *Escherichia coli* is that of an adsorption isotherm. Tested on six different bacteria, the maximum amounts of CTAB adsorbed showed variations from one organism to another.

When cells of *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli* or *Salmonella pullorum* are suspended in water, the ultra-violet spectrum of the supernatant liquid has a maximum absorption at a wave-length of 260 mμ. The height of this maximum is greatly increased when the cells are suspended in CTAB solutions instead of water. Free purines and pyrimidines contribute to this maximum.

There is a parallel relationship between the leakage of 260 mμ.-absorbing material, glutamic acid and inorganic phosphorus from two CTAB-treated Gram-positive bacteria, when their release is followed during the initial rapid phase of leakage. There is a similar relationship for *Esch. coli*, although no glutamic acid is released. Release of cell constituents from CTAB-treated *Staph. aureus* continues slowly for some time after the initial process and is accompanied by a decrease in dry weight of the cells and a gradual change from positive to negative in their Gram-staining reaction. The rate of release of cellular constituents was increased by raising the temperature or by treatment with high concentrations of CTAB.

Treatment of suspensions with sufficient CTAB to sterilize them released amounts of cell constituents comparable to those released by placing the cells in boiling water. When smaller amounts of CTAB were used, a quantitative relationship was found between the amount of CTAB present, the proportion of cells killed and the amount of 260 mμ.-absorbing material released.

Many surface-active agents are bactericidal and various explanations have been proposed to account for this action. Baker, Harrison & Miller (1941) suggested that detergents disorganize the cell membrane and denature certain proteins essential to metabolism and growth. Kuhn & Bielig (1940) observed that germicidal concentrations of some detergents correspond to concentrations effecting denaturation of proteins. Hotchkiss (1944, 1946) found that treatment of bacterial cells with certain surface-active compounds in bactericidal concentrations released nitrogenous and phosphorus-containing substances. Gale & Taylor (1947) demonstrated that the nitrogenous substances released included the free amino-acids concentrated within the cells of *Strep. faecalis*; Mitchell & Crowe (1947) showed that this action was accompanied by an alteration in the surface structure of the cells, as shown by the electron microscope. By growing *Staph. aureus* and *Pseudomonas fluorescens* on media containing radioactive phosphorus (P^{32}), Salton (1950) showed that there was an increased leakage of P^{32} into the suspending fluid when CTAB was present. The release of purines and pyrimidines from *Staph. aureus* treated with CTAB was reported by Salton & Alexander (1950).

The inhibition of bacterial metabolism by surface-active compounds was interpreted by Hotchkiss (1946) as evidence against the idea that an essential, detergent-sensitive enzyme exists. On the other hand, Knox, Auerbach, Zarudnaya & Spirtes (1949) showed that cell-free preparations of certain enzymes from *Esch. coli* are inhibited at detergent/protein ratios which are bactericidal for the intact cells. They concluded that the specific inhibition of detergent-sensitive enzymes, such as the lactic acid oxidase, could account for metabolic inhibition, cell death and increased permeability observed in bacteria treated with bactericidal amounts of the cationic detergents.

The present communication deals with the adsorption of CTAB by bacteria, the disorganization of their cell membranes, and the subsequent release of certain cellular constituents. Cytological studies of the cellular disorganization were carried out in parallel and are reported by Salton, Horne & Cosslett (1951).

METHODS

Organisms. Staph. aureus and *Proteus vulgaris* investigated by Salton (1950); *Strep. faecalis* ST (N.C.T.C. no. 6782); a strain of *Bacillus pumilus* and *Esch. coli* H (kindly given by Dr E. F. Gale); *Salm. pullorum* (kindly given by Dr R. Coombs) were used in these investigations.

Medium, conditions of culture and harvesting. The organisms were grown in a medium consisting of *c.* 3 % tryptic digest of casein, 0·1 % Marmite and 1 % glucose, at an initial pH 7·4–7·6. Roux bottles of medium inoculated with *c.* 10 ml. of overnight culture were incubated for 16 hr. at 37°. The cells were harvested from the liquid medium by centrifugation, washed three times with distilled water on the centrifuge and finally suspended in distilled water to give a suspension containing *c.* 15 mg. dry-weight bacteria/ml.

Dry-weight determinations. The dry weights of the washed bacterial suspensions were determined directly, by drying 1 ml. samples of the dense suspensions to constant weight in an air-oven at 105°.

Adsorption of CTAB. The amount of CTAB adsorbed was determined by adding bacterial suspensions to solutions containing known amounts of CTAB, allowing them to stand at 20° for 15 min., removing the cells by centrifugation and estimating the residual CTAB in the supernatant liquid by the method of Salton & Alexander (1949).

Release of cellular constituents and their estimation in the supernatant fluid. The release of cellular constituents was examined after three types of treatment: (1) cells suspended in distilled water; (2) cells suspended in CTAB solutions; (3) cells pipetted into distilled water at *c.* 100° and then held at that temperature for 10 min. (referred to subsequently as 'boiled' cells). In each case the final suspension density was *c.* 1·5 mg. dry-weight cells/ml. Samples from suspensions were centrifuged for 5 min. to remove most of the cells, and the supernatant finally cleared by further centrifugation. The cell-free supernatants were then analysed for released constituents. For the estimation of some constituents (glutamic acid, purines, pyrimidines) it was necessary to concentrate the supernatant liquids by evaporation to dryness *in vacuo* and to re-dissolve the residues in small volumes of distilled water.

The following methods were used in estimating quantities of released cellular constituents:

(*a*) Ultra-violet spectra were studied by use of the Beckman photoelectric spectrophotometer.

(*b*) Inorganic phosphorus content was estimated by the method of Fiske & Subbarow (1925). Total phosphorus was determined by the procedure of Umbreit, Burris & Stauffer (1945).

(*c*) Pentose was determined by the method of Mejbaum (1939). D-Ribose was used as the standard for a calibration curve.

(*d*) Glutamic acid was estimated by the amino-acid decarboxylase method of Gale (1945).

(*e*) Purines, pyrimidines and derivatives. Paper chromatography was used for the identification and estimation of the purines, pyrimidines and derivatives in the supernatants from bacterial suspensions. These were concentrated and volumes of not more than 0·1 ml. then placed on Whatman filter-paper No. 1, according to the method of Hotchkiss (1948). Of the solvent systems tried, *n*-butanol saturated with water and 5 % (v/v) formic acid gave the best separations. Chromatograms were run with guanine, adenine, uracil and cytosine as markers. The locations of substances on the chromatograms were found by the method of Markham & Smith (1949). For quantitative estimations, the areas of filter-paper corresponding with spots on the developed chromatogram were cut out, extracted with 4 ml. distilled water and estimated at neutral pH in the Beckman spectrophotometer. Filter-paper blanks were obtained by cutting out adjacent areas of equal size as recommended by Hotchkiss (1948). A check on recovery was obtained by running known amounts of purines and pyrimidines and extracting from the chromatograms.

Plate-count estimation of surviving cells. Samples of bacterial suspensions (1·0 ml.) were withdrawn at intervals and transferred to 99 ml. sterile tap water. Suitable dilutions were then plated out, in duplicate, in the growth medium containing 2 % agar and the colonies counted after 48 hr. incubation at 37°.

RESULTS

Adsorption of CTAB by bacteria

The amount of CTAB taken up by *Staph. aureus* and *Esch. coli* is expressed in Fig. 1 as a function of the CTAB concentration in the supernatant. The results suggest typical adsorption isotherms. Similar curves were obtained with *Strep. faecalis*, *B. pumilus*, *Salm. pullorum* and *Pr. vulgaris*. Marked agglutination of the suspensions was observed in the region of maximum CTAB uptake. The level of maximum CTAB adsorption varies somewhat from one organism to another. Table 1 shows maximum amounts of CTAB adsorbed and the weight ratios of bacteria:adsorbed CTAB, for a number of bacterial species.

Release of ultra-violet absorbing material and identification of some of its components

An examination of the ultra-violet spectra of supernatants from suspensions of *Staph. aureus* in distilled water and solutions containing 90μg. CTAB/ml.,

Table 1. *Comparison of maximum amounts of cetyltrimethylammonium bromide adsorbed by various bacteria*

Organism	CTAB adsorbed (μg. CTAB/mg. dry-wt. bacteria)	Weight ratio Bacteria:CTAB
Staph. aureus	320	3:1
Strep. faecalis	430	2·3:1
B. pumilus	600	1·7:1
Esch. coli	420	2·4:1
Salm. pullorum	340	3:1
Pr. vulgaris	355	2·8:1

revealed a maximum absorption at a wave-length of 260 mμ. A marked increase in the 260 mμ. absorption of the supernatant from CTAB-treated cells was observed. Fig. 2 shows the ultra-violet spectra of these supernatant fluids. Spectra of supernatants from untreated and CTAB-treated *Strep. faecalis*, *Esch. coli* and *Salm. pullorum* also showed maxima at 260 mμ., with marked increases in the 260 mμ. absorption when cells had been treated with 90 μg. CTAB/ml. The absorption of the supernatants at 260 mμ. obeyed Beer-Lambert's Law up to *c.* 1·2 optical density units.

Paper chromatography of concentrated supernatants from untreated and CTAB-treated suspensions of *Staph. aureus* showed the presence of a number of components which contribute to the ultra-violet maximum at 260 mμ. Guanine, adenine and uracil were found in the supernatants from untreated suspensions, while guanine, adenine, uracil and adenine riboside were the main components in the supernatant from the CTAB-treated cells. The identification of the free bases guanine, adenine and uracil was confirmed by comparing the spectra of eluted supernatant spots with the spectra of recovered markers and standard solutions of the appropriate bases. The presence of adenine riboside was established by elution from the chromatogram, hydrolysis in N-HCl for 1 hr. and running the hydrolysate on a chromatogram; only one spot, corresponding to adenine, then appeared on the chromatogram. Quantitative data on the release of adenine and uracil which appeared to be present in greater amounts than the other components are given below.

Release of 260 mμ.*-absorbing material relative to other cellular constituents*

The release of glutamic acid (known to be concentrated as the free amino-acid in the internal environment of Gram-positive bacteria; Taylor, 1947) was followed concurrently with the appearance of 260 mμ.-absorbing material and inorganic P. The parallel relationship between the release at 20° of all three cellular constituents from *Staph. aureus* treated with 90 μg. CTAB/ml. is shown in Fig. 3. Similar leakage curves were also found with CTAB-treated *Strep. faecalis* and *Esch. coli*, although no glutamic acid was released from the latter organism. Untreated suspensions of the three bacteria showed a steady release of 260 mμ.-absorbing material, but there appeared to be no significant leakage of inorganic P and glutamic acid. The data for the leakage from untreated and

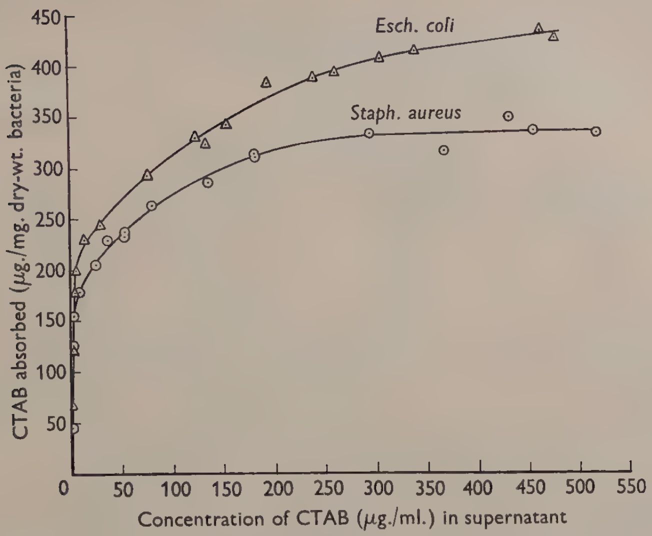

Fig. 1. Adsorption of CTAB by *Staph. aureus* and *Esch. coli* plotted as a function of the CTAB concentration in the supernatant fluid.

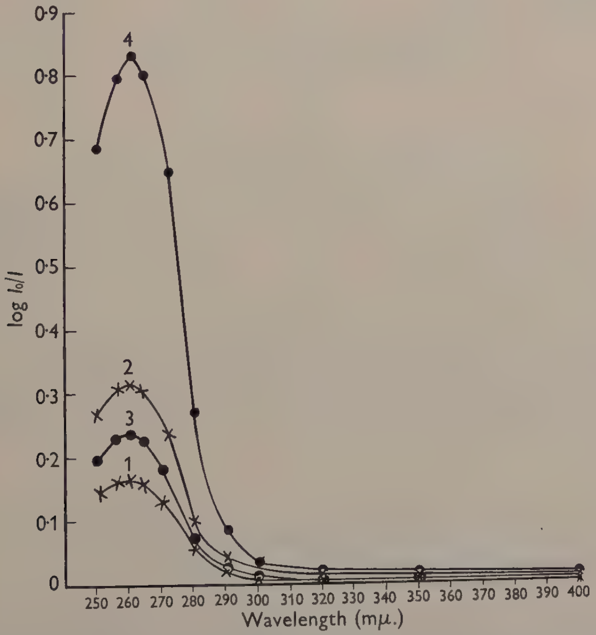

Fig. 2. Ultra-violet spectra of supernatants from *Staph. aureus* suspensions (1·5 mg. dry-wt. cells/ml.) held at 20°. ×——×, distilled water: curve 1, initial sample; curve 2, 180 min. ●——●, treated with 90μg. CTAB/ml.; curve 3, 5 min. exposure; curve 4, 180 min. exposure.

CTAB-treated suspensions of the three organisms are shown in Table 2. Both untreated and treated cells of *Staph. aureus* and *Strep. faecalis* stained Gram-positive throughout the course of the experiment.

Table 2. *Release of cellular constituents from untreated and CTAB-treated bacteria at 20°*

A, 260 mμ.-absorbing material log $[I_0/I]$. B, inorganic phosphorus (μg./mg. dry wt. cells). C, glutamic acid (μg./mg. dry wt. cells)

		Time (min.)						
Organism		0	5	10	30	60	120	180
		Quantities of cellular constituents released						
*Staph. aureus**								
Untreated	A	0·05	—	—	0·07	0·10	0·14	0·19
CTAB-treated, 90μg./ml.	A	—	0·30	0·36	0·52	0·71	0·86	0·90
	B	—	0·9	1·3	2·3	3·0	3·4	3·6
	C	—	6·1	7·7	12·6	15·6	—	19·2
*Strep. faecalis**								
Untreated	A	0·05	—	—	0·06	0·07	—	0·10
CTAB-treated, 45μg./ml.	A	—	·50	—	0·59	0·65	—	0·73
	B	—	3·1	—	3·3	3·4	—	3·7
	C	—	9·6	—	11·2	11·9	—	14·0
Esch. coli†								
Untreated	A	0·07	—	—	0·09	0·10	0·12	0·14
CTAB-treated, 90μg./ml.	A	—	0·30	0·34	0·42	0·49	0·57	0·62
	B	—	0·5	0·6	0·8	1·0	1·2	1·3

* Dry wt., 1·5 mg./ml. suspension.
† Dry wt., 1·4 mg./ml. suspension.

Effect of temperature on the release of cellular constituents from Staphylococcus aureus

When *Staph. aureus* cells were treated with 90 μg. CTAB/ml. at 0, 20 and 30°, appreciable differences in the rates of release of cellular constituents were observed. Plate-count estimations of survivors revealed 99·99 % killing in 5 min. at both 20 and 30°. The initial rates of release of 260 mμ.-absorbing material and inorganic P from CTAB-treated cells were determined over periods of 200, 40 and 20 min. at 1, 20 and 30° respectively. Control rates of release of 260 mμ.-absorbing material from untreated cells were determined over 180 min. at the three temperatures. The data are summarized in Table 3.

Comparison of the amounts of cellular constituents released from CTAB-treated cells with those from 'boiled' cells

On exposure of cells of the three organisms *Staph. aureus*, *Strep. faecalis* and *Esch. coli* to 90 μg. CTAB/ml. at 20° for 180 min., virtually all of the cells were killed (99·99 % mortality). Under these conditions, the amounts of constituents released into the suspending fluid were observed to approach maxima (e.g. Fig. 3 for *Staph. aureus*). Analyses of supernatants from CTAB-treated bacteria revealed quantities of cellular constituents comparable to the amounts released when suspensions were pipetted into water held in a boiling water-bath. The

Table 3. *Influence of temperature on the rate of release of cellular constituents from* Staph. aureus

Temp. (° C.)	Rate of release of constituents: Control, 260 mμ.-absorbing material*	Rate of release of constituents: Treated with 90μg. CTAB/ml.: 260 mμ.-absorbing material*	Rate of release of constituents: Treated with 90μg. CTAB/ml.: Inorganic phosphorus†
0	0·085	0·880	0·005
20	0·551	7·615	0·056
30	0·744	18·950	0·122

* Rate expressed as $10^3 \times \log I_0/I$/mg. dry wt. bacteria/min.

† Rate expressed as μg. phosphorus/mg. dry wt. bacteria/min.

Table 4. *A comparison of the quantities of cellular constituents released from CTAB-treated bacteria with those from 'boiled' cells*

A, untreated suspensions held for 180 min. at 20°. B, suspensions treated with 90 μg. CTAB/ml. for 180 min. at 20°. C, 'boiled' cells.

	Staph. aureus			*Strep. faecalis*			*Esch. coli*		
	A	B	C	A	B	C	A	B	C
Constituents estimated	μg. released constituent/mg. dry wt. bacteria								
Inorganic phosphorus	0	3·6	3·5	0	3·6	4·1	0	1·1	1·0
Total phosphorus	0	5·4	5·5	0	5·1	5·2	0	1·9	3·3
Pentose	0	4·9	6·6	0	7·3	7·7	0	4·8	13·0
Glutamic acid	0	26·6	27·8	0	10·7	11·2	0	0	0
Adenine									
(*a*) Unhydrolysed supernatant	0·4	1·8	—	—	—	—	—	—	—
(*b*) Hydrolysed supernatant, N-HCl, 1 hr. at 100°	—	2·4	2·8	—	—	—	—	—	—
Uracil	0·6	1·4	0·9	—	—	—	—	—	—

amounts of adenine and uracil contributing to the leakage of 260 mμ.-absorbing material from untreated suspensions of *Staph. aureus* were estimated. The data are shown in Table 4.

Effect of CTAB concentration on the release of 260 mμ.*-absorbing material*

The amounts of 260 mμ.-absorbing material appearing in the suspending fluids were estimated for a range of CTAB concentrations from 22·5 to 900 μg./ml. Samples were taken after 5, 30 and 180 min. exposure at 20° to the CTAB solutions. At least four estimations were made for each CTAB concentration and sampling-time. Fig. 4 shows the results obtained with *Staph. aureus* and illustrates those obtained also with *Strep. faecalis* and *Esch. coli*; the complete data are recorded in Table 5.

The data show that leakage of 260 mμ.-absorbing material was a slower process for *Staph. aureus* and *Esch. coli* than for *Strep. faecalis* when the CTAB

Table 5. *Effect of CTAB concentration on the release of* 260 mμ.*-absorbing material from various organisms*

CTAB concentration (μg./ml.)	*Staph. aureus*			*Strep. faecalis*			*Esch. coli*		
	5 min.	30 min.	180 min.	5 min.	30 min.	180 min.	5 min.	30 min.	180 min.
	260 mμ.-absorbing material released (log (I_0/I)/mg. dry wt. cells)								
22·5	0·017	0·035	0·182	0·182	0·220	0·300	0·036	—	—
45	0·036	0·090	0·420	0·344	0·378	0·435	0·069	0·096	0·124
90	0·120	0·325	0·450	0·440	0·445	0·465	0·178	0·228	0·325
135	0·250	0·448	0·450	0·454	0·456	0·457	0·231	—	—
180	0·355	0·453	—	0·460	0·456	0·453	0·272	0·321	0·323
270	0·430	0·450	0·450	0·455	0·456	0·453	0·281	0·325	0·325
450	0·375	0·400	0·424	0·403	0·410	0·414	0·257	0·325	>0·500
630	0·345	0·370	0·447	0·356	0·358	0·377	0·326	0·384	>0·500
900	0·438	0·480	0·504	0·356	0·375	0·400	0·336	0·440	>0·500

concentrations were below *c.* 200 μg./ml. With *Strep. faecalis* the release was virtually complete in 5 min. for CTAB concentrations between 90 and 270 μg./ml. As the CTAB concentration was increased above 270 μg./ml., the 260 mμ. absorbing material appearing in the supernatants from the three bacteria fell to a minimum and rose again. These discontinuities appeared in the region of maximum CTAB uptake by the cells. To test several possibilities which may account for the discontinuities, *Staph. aureus* was treated with 270 μg. CTAB/ml. for 30 min. Under these conditions most of the CTAB was adsorbed, concentrations of residual CTAB were found to be less than 10 μg./ml. and the release of 260 mμ.-absorbing material had ceased (see Fig. 4). When CTAB was added to give final concentrations in the supernatant of 100–500 μg. CTAB/ml., no change in the optical density or absorption maximum could be detected. Further, the addition of CTAB to supernatants showed no traces of turbidity or precipitate formation. The addition of more CTAB to suspensions previously treated with 270 μg. CTAB/ml. resulted in a 5 % decrease in the amount of 260 mμ.-absorbing material found in the supernatant. Adsorption of some of the 260 mμ.-absorbing material on the CTAB-saturated cells, would therefore partly account for the fall to a minimum shown in Fig. 4.

Relationship between bactericidal effects, amount of CTAB and release of 260 mμ.*-absorbing material*

The bactericidal activity of CTAB was followed concomitantly with the release of 260 mμ.-absorbing material from suspensions of *Staph. aureus*, *Strep. faecalis*, *Esch. coli* and *Salm. pullorum*. Samples for estimating surviving bacteria and 260 mμ.-absorbing material released were taken after 5 and 30 min. exposure to CTAB at 20°. Estimations of viable cells from untreated suspensions held for 30 min. at 20° showed no differences from the initial populations. The bactericidal effects of 22·5 and 45 μg. CTAB/ml. on known weights of the four organisms, together with the amounts of 260 mμ.-absorbing material released, are shown in Table 6.

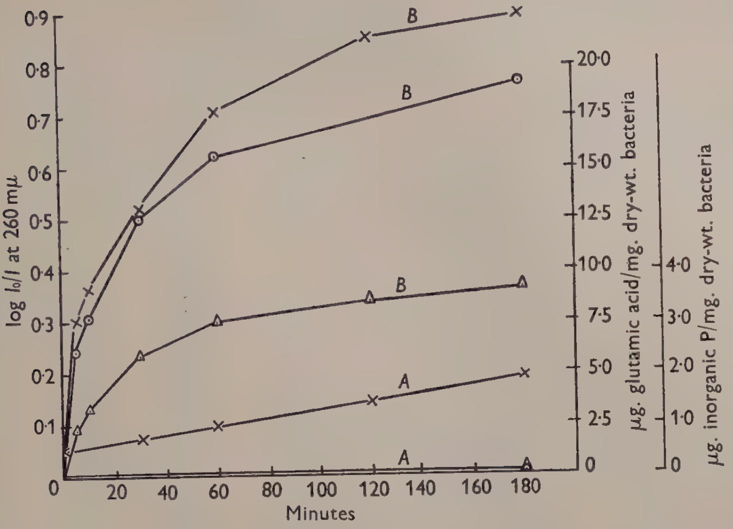

Fig. 3. The course of release of cellular constituents from *Staph. aureus* (1·5 mg. dry-wt. cells/ml.) suspensions at 20°, the amount of constituents appearing in the supernatant being plotted against time. ×——×, 260 mμ.-absorbing material; ⊙——⊙, glutamic acid; △——△, inorganic P; *A*, cells suspended in water; *B*, cells suspended in a solution containing 90 μg. CTAB/ml.

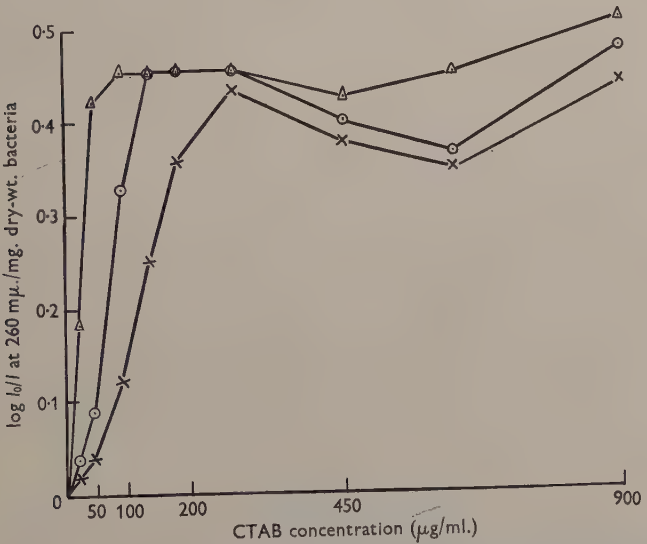

Fig. 4. Effect of CTAB concentration on the release of 260 mμ.-absorbing material from *Staph. aureus* at 20°. The amount of 260 mμ.-absorbing material released into the suspending fluid was estimated for: ×——×, 5 min. exposure; ⊙——⊙, 30 min. exposure; △——△, 180 min. exposure.

Table 6. *Relationship between percentage cells killed, amount of CTAB and release of* 260 mμ.*-absorbing material at* 20°

Organism	Dry-wt. bacteria (mg./ml.)		22·5 μg. CTAB/ml.		45 μg. CTAB/ml.	
			5 min. exposure	30 min. exposure	5 min. exposure	30 min. exposure
Staph. aureus	1·8	Killed (%)	24	35	50	62
		260 mμ. material released (log I_0/I)	0·036	0·061	0·071	0·116
Strep. faecalis	1·5	Killed (%)	43	55	85	97
		260 mμ. material released (log I_0/I)	0·255	0·315	0·508	0·571
Esch. coli	1·4	Killed (%)	16	28	31	57
		260 mμ. material released (log I_0/I)	0·049	0·069	0·094	0·137
Salm. pullorum	1·5	Killed (%)	26	31	54	62
		260 mμ. material released (log I_0/I)	0·156	0·175	0·314	0·347

Autolysis initiated by CTAB treatment

When the appearance of cellular constituents in the suspending fluid was followed over an extended period, autolytic breakdown of the cells did contribute to the amount of materials found in the supernatants, as suggested by the data of Hotchkiss (1944, 1946). The appearance of 260 mμ.-absorbing material and pentose in the suspending fluid from *Staph. aureus* treated with 90 μg. CTAB/ml. at 30° was followed together with the Gram-stain reaction of the cells and changes in the dry weight of centrifuge-deposited untreated and CTAB-treated bacteria (i.e. cells freed from the supernatant fluid). These measurements are recorded in Table 7.

The results show an initial leakage process which is completed in 3–4 hr. This is then followed by a slower release of constituents, accompanied by a gradual change in staining from Gram-positive to Gram-negative and a progressive decrease in dry-cell mass. The results with this organism therefore appear to differ from those of Hotchkiss (1946) in that autolytic breakdown of the cells does not appear to contribute significantly to the amounts of cellular constituents released within the first few hours of exposure of the cells to CTAB.

DISCUSSION

Bacterial cells have a high affinity for CTAB molecules. For the six organisms studied, the amount of CTAB adsorbed per cell, at saturation levels, was found to be more than the amount corresponding to a closely packed monolayer of the detergent. CTAB molecules occupy an area of *c.* 45 A.^{2} (Friend & Schulman, private communication). The amount of CTAB taken up at saturation of *Staph. aureus* would, therefore, occupy an area *c.* 15 times the cell surface area (assuming a sphere of 1 μ. in diameter). The corresponding ratio for *B. pumilus* is

Table 7. *Release of cell constituents, changes in dry weight and Gram-stain of untreated and CTAB-treated suspensions of* Staph. aureus *held at* 30°

A, untreated cells. B, cells treated with 90 μg. CTAB/ml.

	260 mμ.-material log $[I_0/I]$		Pentose (μg./ml.)		Dry wt. (mg./10 ml. sample)		Gram stain (approx. % Gram-positive)	
Time	A	B	A	B	A	B	A	B
0 min.	0·12	0·12	0	0	19·7	19·7	100	100
5 min.	—	0·38	—	—	—	19·4	100	100
10 min.	—	0·45	—	3·2	—	19·5	100	100
20 min.	—	0·64	—	4·2	—	18·5	100	100
40 min.	—	0·96	—	6·3	—	18·3	100	100
60 min.	0·26	1·12	0·9	8·1	19·6	17·5	100	100
120 min.	0·40	1·31	1·0	9·0	19·2	16·6	100	100
180 min.	0·56	1·42	—	9·8	—	16·8	100	100
4 hr.	0·70	1·44	—	9·7	18·9	16·3	100	100
6 hr.	0·94	1·54	—	—	—	15·9	100	90
8 hr.	—	1·64	—	12·3	—	15·6	100	75
10 hr.	1·30	1·76	2·0	—	18·4	15·3	100	50
12 hr.	—	1·84	—	—	18·6	15·1	100	25
13 hr.	1·57	1·92	2·2	15·0	—	15·1	100	10
22 hr.	—	—	—	21·0	18·4	14·8	100	0
28 hr.	—	2·40	—	24·3	—	13·3	100	0

c. 18 times the cell-surface area, assuming cell dimensions of 0·8 μ. width, 3 μ. length. This suggests that CTAB is adsorbed at sites additional to those available at the cell surface, if it be all adsorbed as a monolayer. Alexander & McMullen (1949) and McQuillen (1950) also suggested the possibility of CTAB adsorption at sites within the bacterial cytoplasm. Valko & Dibblee (1944) reported that adsorption of dimethyldodecylbenzylammonium chloride by *Esch. coli* corresponded to an equivalent weight of the bacterium of *c.* 600, an equivalent weight about the same as that of many animal proteins. It would seem, therefore, that both surface and internal components of the bacterial cell are capable of titrating with cationic detergents. McQuillen (1950) suggested the possibility of some detergent being held as concentric shells around the bacterial cell, but his electrophoresis data afforded no confirmation of this. However, the agglutination of bacterial suspensions at CTAB-saturation levels indicates that the surface of the cells has become hydrophobic, a phenomenon analogous to the agglutination of red blood cells with tannic acid, observed by Schulman & Rideal (1937).

As Anson (1939) pointed out, any study of killing and lysis of bacteria by detergents should include a series of concentrations in order to determine the chain of reactions that occur in the organism. From the work reported here, it is possible to appreciate something of the course of events when bacteria are exposed to the cationic detergent CTAB. On exposure of various species to bactericidal amounts of CTAB below that required for 99·99 % killing, the leakage of 260 mμ.-absorbing material in 5 min. is proportional to the percentage of cells killed. These observations are in agreement with the quantitative relationship between the proportion of cells killed by different amounts of tyrocidin and release of amino-acids reported by Gale & Taylor (1947). When cell suspensions are treated with sufficient CTAB (*c.* 90 μg. CTAB/1·5 mg. dry-weight cells) to kill 99·99 % of the cells within 5 min., the rapidity with which cellular constituents appear in the suspending fluid varies from one organism to another. Where the release of cellular constituents was found to be relatively slow (e.g. 3–4 hr. with *Staph. aureus*) temperature affected the rate of leakage of materials into the suspending fluid. Cellular constituents were also more rapidly released when suspensions were treated with amounts of CTAB above those exhibiting maximum killing.

The results reported here support the view that there is a primary process of cell-solute release on treatment of bacteria with CTAB. Autolytic breakdown of the cells does not appear to make a significant contribution to the materials found in the suspending fluid during this initial leakage. This conclusion is supported by the following experimental observations: (1) the parallel relationship between the release of free amino-acid, inorganic P, purines and pyrimidines, etc.; (2) the amounts of cellular constituents released from bacteria exposed to 90 μg. CTAB/ml. for 180 min. at 20° were comparable to the amounts released from 'boiled' cells; (3) a change in the Gram-staining of *Staph. aureus* was not apparent until several hours after the initial leakage of cell solutes (Table 7). However, a study of cell constituents released when suspensions were treated with 90 μg. CTAB/ml. and held for periods of more than 3–4 hr. or treated with CTAB concentrations near and above the level required for cell saturation, suggested that processes such as autolytic breakdown, adsorption of released materials on to CTAB-saturated cells and solubilization of cell materials in detergent micelles were involved. Autolysis of bacteria associated with treatment of cells in low and high concentrations of surface-active agents has been well established in previous studies. This phenomenon was reviewed by Dubos (1945), and Hotchkiss (1946) has contributed further information. The possibility of physical solubilization of cellular materials in bacteria + detergent studies has been suggested by the work of Stacey (1949).

That treatment of bacteria and yeasts with detergents results in cellular disorganization is further supported by cytological evidence of cytoplasmic shrinkage, cell rupture, etc. (Dyar, 1947; Mitchell & Crowe, 1947; Meisel & Umanskaya, 1949; Salton *et al.* 1951).

Knox *et al.* (1949) believe that specific inhibition of a detergent-sensitive enzyme, such as the lactic acid oxidase of *Esch. coli*, can account for the metabolic inhibition, cell death and increased permeability observed in bacteria treated with cationic detergents. It does not seem to be necessary to invoke the inhibition of a detergent-sensitive enzyme as being the primary cause of cell death. Indeed, if there were a chain of reactions initiated by a primary metabolic inhibition, a greater time lag before the observation of secondary effects such as the release of cellular constituents might well be expected. However, it appears that leakage of cell solutes, inhibition of sensitive enzymes and cytological damage can all be observed simultaneously with cell death. The interaction between detergent molecules and cell components (e.g.

proteins, enzymes, lipo-proteins etc.) as such, could account for cell death, leakage of solutes, etc. Studies of the penetration of protein and lipo-protein mono-layers by surface-active compounds, discussed by Schulman (1951), appear to have some relevance to the action of these agents on the permeability barrier of bacteria. It would seem that there is little evidence which disagrees with the original hypothesis of cell membrane disorganization proposed by Baker *et al.* (1941). However, it is yet too early to speculate on the primary site or sites of detergent action. A clearer picture will undoubtedly emerge when more is known of the chemical nature of the surface components and outer envelope of the bacterial cell.

This work was performed during the tenure of a Studentship, for which I am indebted to the Commonwealth Scientific and Industrial Research Organization of Australia. I also wish to express my sincere thanks to Prof. A. E. Alexander, Dr J. H. Schulman and Dr E. F. Gale for their interest, encouragement and helpful criticism throughout the course of the investigations.

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Electron Microscopy of Bacteria treated with Cetyltrimethylammonium Bromide

BY M. R. J. SALTON, R. W. HORNE AND V. E. COSSLETT

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SUMMARY: When viewed in the electron microscope, cells of *Staphylococcus aureus*, *Streptococcus faecalis* and *Escherichia coli* changed markedly when suspended in cetyltrimethylammonium bromide (CTAB) solutions instead of distilled water; the cytoplasm contracts away from the cell wall. The proportion of cells showing such contraction, in the case of *Esch. coli*, can be correlated with the proportion of cells killed by the treatment. High concentrations of CTAB appear to strip off the cell envelopes.

Direct evidence of cytolytic damage in detergent-treated bacteria was first provided by the electron micrographs of Mitchell & Crowe (1947). They observed rupture of the cell wall concomitantly with the leakage of amino-acids from tyrocidin-treated *Strep. faecalis*. Dyar (1947) used cetylpyridinium chloride as a mordant, before staining bacteria and yeast with congo red and methylene blue. He noted that the cytoplasm of the cell appeared to have shrunk away from the cell wall. Meisel & Umanskaya (1949) contributed further evidence of cytolytic damage resulting from treatment of a yeast with cetyl-zephirol; they observed contraction of the protoplast and its separation from the cell envelope.

The present paper presents evidence of cytological damage obtained by examination of CTAB-treated bacteria in the electron microscope. These observations have been made concomitantly with the studies of cell death, leakage of cell solutes and detergent adsorption reported by Salton (1951).

METHODS

The three organisms studied were *Staph. aureus*, *Strep. faecalis* ST (N.C.T.C. no. 6782) and *Esch. coli* H. Suspensions of the organisms, containing *c.* 1·5 mg. dry-weight cells/ml., were treated with concentrations of CTAB ranging from 45 to 900 μg./ml. The cells deposited on centrifuging samples of untreated and CTAB-treated bacteria were resuspended in distilled water, after the supernatants containing the released cell solutes had been decanted. Drops of dilute suspensions were placed on standard electron microscope specimen grids covered with nitrocellulose film. Mounted specimens were dried in a desiccator and shadowed with gold-palladium alloy (60 %/40 %) by shadowcasting technique (Wyckoff, 1949), at an angle of *c.* 45 degrees from the plane of the supporting film. A large number of untreated and treated bacteria were examined in the Siemens electron microscope. Most of the observations were made at direct magnifications of 8000–12000. The contrast between the outer cell wall and the supporting film 'background' was increased by using 20 μ. objective apertures.

RESULTS

When suspensions of *Staph. aureus* cells in distilled water were held for periods of 30 min. at 20°, no change in the appearance of the cells was observed; Pl. 1, fig. 1, shows the typical appearance of such cells. The cytoplasm in these cells is diffuse at its periphery near the cell wall. Exposure of *Staph. aureus* (*c.* 1·5 mg. dry wt./ml.) to 90 μg. CTAB/ml. for 30 min. at 20° results in death of 99 % of the cells and the release of an appreciable fraction of the cell solutes into the suspending fluid (Salton, 1951). Such cells showed a marked change in appearance, and shrinkage of the cytoplasm from the cell wall is clearly shown in Pl. 1, figs. 2, 3. *Staph. aureus* suspensions exposed to 90 μg. CTAB/ml. for 28 hr. at 30° were Gram-negative and had decreased in dry weight by more than 30 %. Pl. 1, fig. 4, shows the partially digested appearance of such cells and the absence of cell walls. Treatment of *Staph. aureus* suspensions with 900 μg. CTAB/ml. for 30 min. at 20° stripped off the cell envelope, either partially as shown in Pl. 2, fig. 5, or completely as in Pl. 2, fig. 6.

In the case of *Strep. faecalis*, untreated cells (Pl. 2, fig. 7) cast little shadow, and therefore seem to lie flatly on the supporting film. As with *Staph. aureus* the cytoplasm is diffuse near the cell wall. When *Strep. faecalis* is treated with 90 μg. CTAB/ml. for 30 min. at 20°, 99 % of the cells are killed and the release of cell solutes into the suspending fluid is complete. The contraction of the cytoplasm revealing the thin outer cell wall is shown in Pl. 2, fig. 8.

Esch. coli cells from distilled-water suspensions are shown in Pl. 3, fig. 9 Treatment of *c.* 1·5 mg. dry weight of this organism with 45 μg. CTAB/ml. for 5 min. at 20° result in the death of *c.* 30 % of the cells and initiates the leakage of cellular constituents. Examination of cell suspensions treated under these conditions showed that in 30–40 % of the cells the cytoplasm had contracted from the cell wall, the remaining 60–70 % of the cells appearing more like the untreated cells; Pl. 3, fig. 10, shows a typical field. Pl. 3, fig. 11, shows that contraction of the cytoplasm had occurred in all cells when *Esch. coli* was exposed to sufficient CTAB (*c.* 90 μg./1·5 mg. dry-weight bacteria) to kill virtually all the cells in 30 min. When treated with 900 μg. CTAB/ml. for 30 min. at 20°, the cell wall of *Esch. coli* appeared to be stripped off (Pl. 3, fig. 12).

DISCUSSION

Our observations are in agreement with earlier reports of cytological damage resulting from detergent treatment of micro-organisms (Mitchell & Crowe, 1947; Dyar, 1947; Meisel & Umanskaya, 1949). From the work of Salton (1951) and our observations it is evident that the damage caused by CTAB to the permeability barrier, resulting in the leakage of cell solutes, is accompanied by a marked contraction of the cytoplasm, which becomes detatched from the cell wall. For *Esch. coli* there is a correlation between the number of cells killed and the proportion of cells in which the cytoplasm has contracted from the cell wall.

On treating bacteria with sufficient CTAB to kill the cells and release cell solutes, cellular disorganization is manifest in the contraction of the cytoplasm

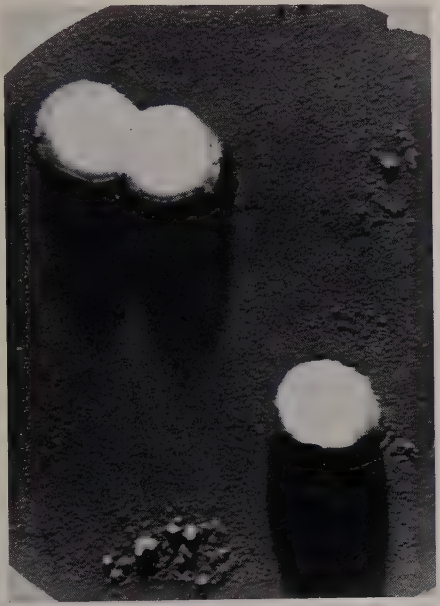

Fig. 1

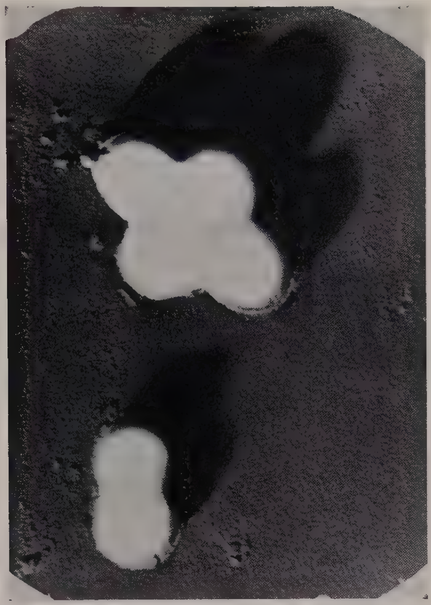

Fig. 2

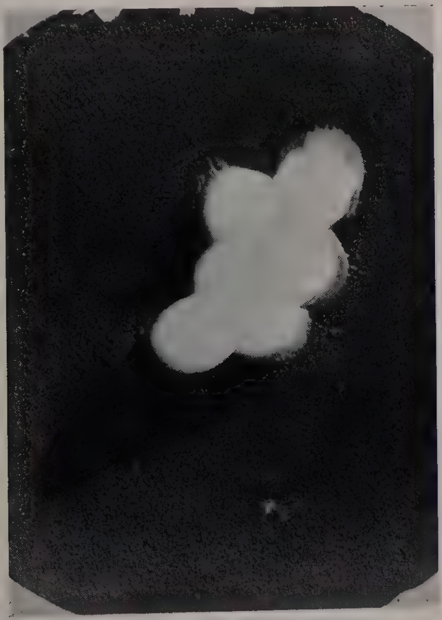

Fig. 3

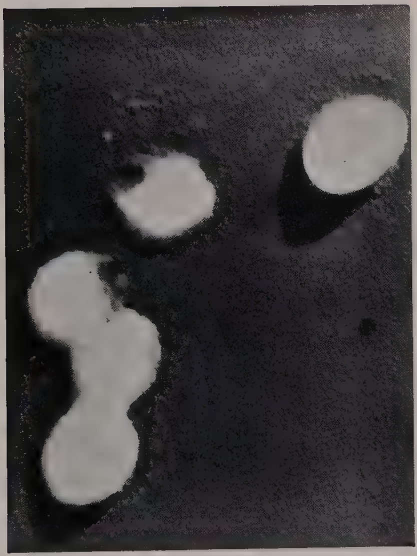

Fig. 4

M. R. J. SALTON, R. W. HORNE & V. E. COSSLETT—ELECTRON MICROSCOPY OF CTAB-TREATED BACTERIA. PLATE 1

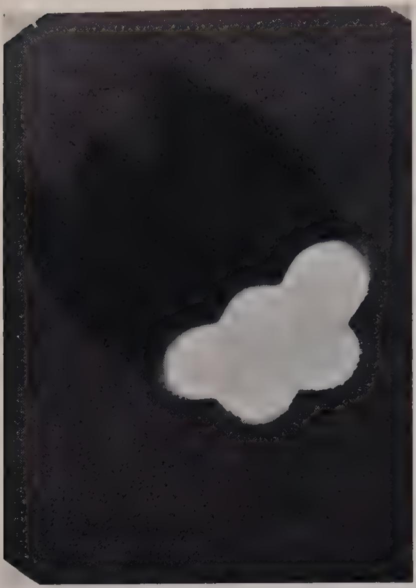

Fig. 5

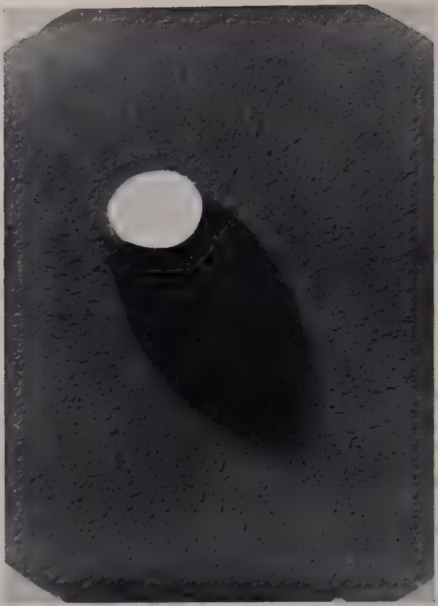

Fig. 6

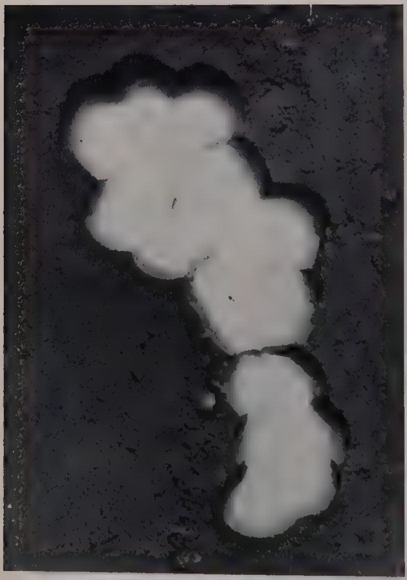

Fig. 7

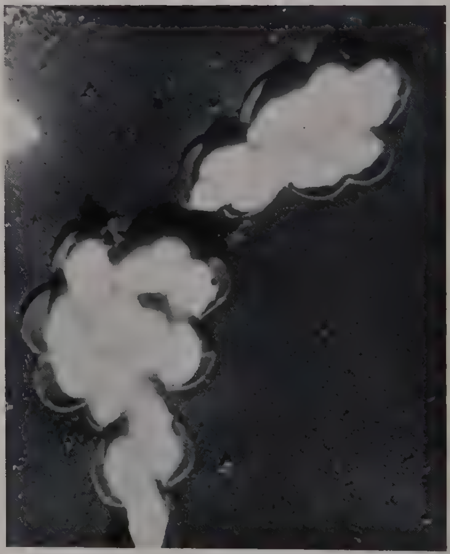

Fig. 8

M. R. J. Salton, R. W. Horne & V. E. Cosslett—Electron microscopy of CTAB-treated bacteria. Plate 2

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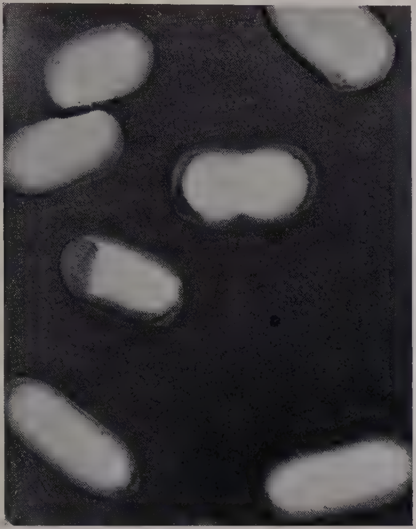

Fig. 9

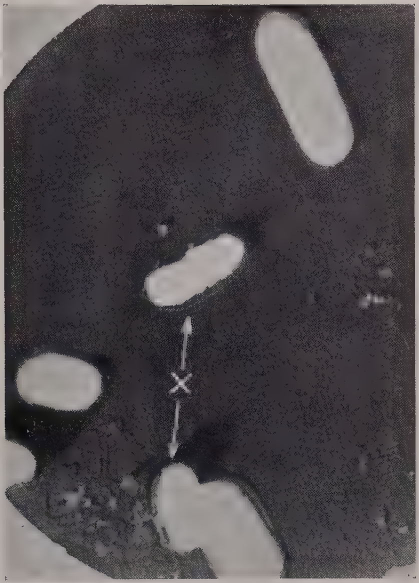

Fig. 10

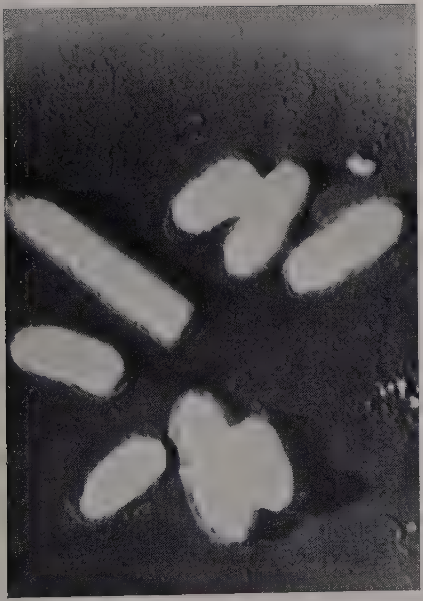

Fig. 11

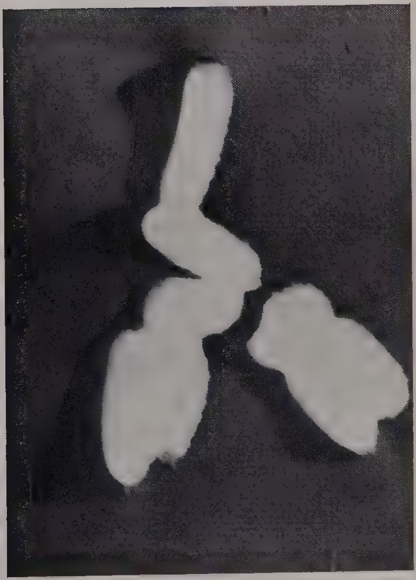

Fig. 12

M. R. J. Salton, R. W. Horne & V. E. Cosslett—Electron microscopy of CTAB-treated bacteria. Plate 3

from the cell wall which can be seen clearly as an outer envelope enclosing the cytoplasm. However, when CTAB-treated cells are allowed to autolyse or when cells are treated with larger amounts of CTAB, the cell wall itself is either partly or completely stripped off. Permeability alterations by CTAB can thus be observed at lower CTAB concentrations than those required to strip off the outer cell envelopes.

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EXPLANATION OF PLATES

All photographs are electron micrographs of preparations shadowed with Au (60 %)–Pd (40 %) alloy.

PLATE 1

Fig. 1. *Staph. aureus*. Distilled water suspension. × 18,000.

Fig. 2. *Staph. aureus* treated with 90 μg. CTAB/ml. for 30 min. at 20°. × 18,000.

Fig. 3. *Staph. aureus* treated with 90 μg. CTAB/ml. for 30 min. at 20°. × 18,000.

Fig. 4. *Staph. aureus* treated with 90 μg. CTAB/ml. for 28 hr. at 30°. × 22,000.

PLATE 2

Fig. 5. *Staph. aureus* treated with 900 μg. CTAB/ml. for 30 min. at 20°. × 18,000.

Fig. 6. *Staph. aureus* treated with 900 μg. CTAB/ml. for 30 min. at 20°. × 18,000.

Fig. 7. *Strep. faecalis*. Distilled water suspension. × 10,500.

Fig. 8. *Strep. faecalis* treated with 90 μg. CTAB/ml. for 30 min. at 20°. × 11,000.

PLATE 3

Fig. 9. *Esch. coli*. Distilled water suspension. × 8,500.

Fig. 10. *Esch. coli* (1·5 mg. dry weight) treated with 45 μg. CTAB for 5 min. at 20°. 'X', indicates cells in which the cytoplasm has contracted from the cell envelope; note that the contracted cytoplasm casts appreciably more shadow. × 8,500.

Fig. 11. *Esch. coli* treated with 90 μg. CTAB/ml. for 30 min. at 20°. × 9,000.

Fig. 12. *Esch. coli* treated with 900 μg. CTAB/ml. for 30 min. at 20°. × 8,500.

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Observations on the Value of the Bordet-Gengou Medium for the Cultivation of *Haemophilus pertussis*

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SUMMARY: No significant improvement has been made on the medium first described for the primary culture of *Haemophilus pertussis* by Bordet & Gengou in 1906. An explanation for this was sought in the experiments described, and it was found to be most probable that potato extract provides optimal concentrations of amino-acids and peptides required for growth. Although serum can be used to replace whole blood in this medium, the latter is essential for maximal growth; for that reason the retention of the term haemophilus is justified.

Many peptones, especially the more digested ones, inhibit growth of *H. pertussis* in concentrations normally used in bacteriological media. In the absence of peptone, meat extract agar can be used as the base of a satisfactory medium, especially when used in conjunction with horse instead of rabbit blood.

In surveying the literature on the cultivation of *H. pertussis* we have not found any record of a considerable series of isolations of the bacillus from cases of the disease in which authors have departed seriously from the original medium of Bordet & Gengou (1906) (cf. Wollstein, 1909; Chievitz & Mayer, 1918; Sugare & McLeod, 1929; Sauer & Hambrecht, 1930; Gardner & Leslie, 1932; Straker & Westwater, 1937). A minor divergence from the original formula which has sometimes been used is the incorporation of *c.* 0·5 % of lactic acid in the potato-extract blood agar, e.g. Leslie & Gardner (1931) following a recommendation of the Danish school (Madsen, 1925). Bailey (1933) recommended a similar modification and maintained that such a medium gave better growth than the original Bordet-Gengou medium (referred to subsequently in this paper as BG medium).

We have seen only one other claim to have prepared a medium for phase I strains which is better than the original, that of Silverthorne & Cameron (1942), who recommended a beef-liver extract blood agar. In our experience, however, this is no better than the standard BG medium. Surprisingly some text-books, e.g. Mackie & McCartney (1949), recommend a medium which differs from the original BG medium in the important respect that it contains peptone. Its success probably depends on the facts that the peptone recommended is one of the least inhibitory (see Table 1), and that in the complete medium it is only present in 0·5 % (w/v) concentration. Pollock (1947) reported that 'the modified (BG) medium most commonly used consists of 30–50% whole blood and potato extract as well as a basis of peptone or tryptic digest of meat'.

It has frequently been observed, although insufficiently emphasized in some text-books, that freshly-isolated phase I strains of *H. pertussis* fail to grow or grow very poorly on nutrient agar even with the incorporation of blood. This is stated in the work of Kristensen (1922), of Sugare & McLeod (1929), of

Kleinschmidt (1931) who observed that recently isolated *H. pertussis* did not grow on Levinthal agar, and of Lawson (1939) who pointed out that smooth phase I *H. pertussis* did not grow on chocolate agar, Fildes agar, blood agar, ascitic agar, or Loeffler's medium.

In our experience, an organism of correct morphology and staining which grows well on BG medium and fails to grow on heated blood nutrient agar ('chocolate') is quite as accurately recognized as a smooth phase I strain of *H. pertussis* as by agglutination. This is in frank contrast with *H. influenzae*, *H. parapertussis* and *H. pertussis* in other phases. So far as we have been able to determine, no one has explained this phenomenon. Pollock (1947) made interesting observations on factors controlling the growth of *H. pertussis* and demonstrated its special sensitivity to unsaturated fatty acids and the significance of serum albumin in protecting the bacterium from the noxious effect of these fatty acids. His work, however, was done with a partly adapted or trained strain of *H. pertussis* which was capable of growing on 10 % blood agar. Further, in our hands the incorporation of activated charcoal in nutrient agar did not promote the growth of very recently isolated strains of *H. pertussis*, as Pollock found that it did that with his adapted strain. Hence his observations do not explain the failure of recently-isolated strains of *H. pertussis* to grow on blood nutrient agar. They may explain the peculiar discrepancy that the fluid medium of Hornibrook (1939, 1940), which has been extensively used to obtain growths of *H. pertussis*, fails when used as a solid agar medium (Verwey, Thiele, Sage & Schuchardt, 1949). According to Pollock, agar itself may contribute unsaturated fatty acids.

The principal objective in our work was to find a medium which would promote more abundant and rapid growth of *H. pertussis* than does BG medium, and which would, therefore, afford a more rapid and constant aid in the diagnosis of whooping-cough. We decided, therefore, to survey each of the ingredients in the BG medium, and to search for an explanation of the peculiar difficulty of growing recently-isolated strains in nutrient agar with added blood. The results of our work may be summarized by stating that for the purpose of growth from small inocula of strains recently isolated ($\frac{1}{2}$–3 weeks), we failed to find any medium which consistently gives better growths than the original BG medium prepared with rabbit or human blood. Our work, however, revealed certain facts with regard to the primary culture of *H. pertussis* which have been insufficiently emphasized.

The original BG medium consisted of a base of potato extract, glycerol and saline, to which 50 % (v/v) whole blood was added. In the original communication rabbit or preferably human blood was recommended. This formula is the basis for most preparations of BG medium to-day, although the proportion of blood is usually only 30 % and bloods from other species (e.g. sheep, horse) are used. It should be observed that the original BG formula does not require any peptone. This is definitely stated (Bordet & Gengou, 1906; footnote to p. 734), but no reason is given other than that there is less profuse growth of saprophytic bacteria in its absence. The relevance of this omission of peptone will be stressed later.

EXPERIMENTAL

At least three strains were tested simultaneously in all experiments, and invariably one or more of these strains had been isolated within a fortnight of the experiment and had undergone at the most two subcultures on BG medium. In this respect there is a sharp contrast with much other work on the subject. Approximately standard inocula were obtained by preparing 1/100 and 1/1000 dilutions of suspensions of just visible turbidity in saline. One loopful of each of the dilutions was used, and it was found that from the more dilute inocula a countable number of discrete colonies grew on suitable media, so that the size and numbers of colonies could be compared. One or more control plates of BG medium were included in every experiment. Each component of the BG medium was then examined to determine its importance.

Glycerol. The value of the glycerol does not appear to be very great. Although normally added to the potato before extraction, addition at a later stage does not appear to be a disadvantage. Increase in concentration beyond 1 % (w/v) in the basal medium produces some inhibition of growth, while its complete elimination causes only slight diminution of growth. In all control plates of BG medium, however, it was retained.

Agar. Variations in media caused by the use of different commercial preparations of agar suggested that some were definitely superior to others. A sample of 'Oxoid' agar (code no. L. 10) prepared by Oxo Ltd. London, from material of New Zealand origin, was satisfactory and used throughout at 1·4 % (w/v) in the completed medium.

Potato extract. In this complex component only the most obvious constituents have been examined. An average analysis of whole potato gives carbohydrate 20 % and protein 2 %. Soluble carbohydrate, protein or protein derivatives and inorganic salts are present in the aqueous extract. The effects of extracts of different parts of the potato, of different varieties of potato, and of different methods of preparation were studied. The following extracts were incorporated in BG medium and gave similar growths: from cortex or centre of young, old and sprouting potatoes or of varieties obtained from different areas. Equally good growth was obtained when the extract was prepared by autoclaving slices after standing in water for 30 min., or by dropping them into boiling water as soon as sliced. Filtration of an extract of autoclaved potato gave as good a result as unfiltered extract containing much insoluble matter. It appears that considerable variations in the preparation of potato extract may be adopted without appreciable effect. There are variations, however, which result in definitely inferior growth. This occurs when the extract is concentrated to half its usual volume, when potato starch solution is substituted for it, or when extracts at temperatures below 55° are sterilized by filtration with Maassen candles. The last procedure, designed to retain active enzymes, yielded extracts which partially inhibited growth and became dark brown on standing. The method of extraction finally adopted was to autoclave thinly-sliced peeled potato at 10 lb./sq.in. for 15 min., with twice its weight of aqueous glycerol (4 %). The extract was then separated by filtration through lint, using moderate pressure, and diluted with 0·6 % saline in accordance with the BG formula.

Observations on the amino-acid content of potato extract

The filtrates and residues obtained both by dialysis and by ultra-filtration using collodion sacs were compared with the original extract. This comparison showed that the growth-promoting material passed through the bag, the dialysate and ultra-filtrate giving results identical with those obtained with the original potato extract. The residuum left after dialysis or ultrafiltration gave poorer growth. The protein and starch present in potato extract appear, therefore, to be relatively unimportant. Although Groot (1946) made an extensive investigation of the amino-acids present in potato protein this examination did not extend to the free amino-acids present in potato juice or extract. As we have not found any account of the amino-acid content of potato extract we examined it by two-way paper chromatography. There was evidence of the presence of many amino-acids, including glutamic and aspartic acids, which was interesting in view of the fact that these two amino-acids have a stimulating effect on the growth of *H. pertussis* (see below). Since an acid-hydrolysate of casein equivalent to about 1 % (w/v) in the basal medium gave growth only slightly inferior to that obtained with potato extract it may be that a collection of amino-acids in certain proportions is responsible for the value of potato extract in the BG medium. It should be mentioned that *H. pertussis* will grow on a medium entirely devoid of potato extract, i.e. containing saline, agar and blood only, although growth is much less good.

Blood

Bordet & Gengou originally suggested the incorporation of 50 % (v/v) human or rabbit blood in their medium. We confirmed that *in their medium* the bloods of these two species are superior to that of the horse. The amount required can be varied between 30 and 50 % (v/v) without appreciable change in the amount of growth; with less than 30 % progressively inferior results were obtained. Defibrinated and oxalated blood were equally effective. It is stressed in some text-books that *H. pertussis* may be differentiated from *H. influenzae* by its inability to grow in primary culture on a heated blood medium. In these cases, however, it is probable that blood nutrient agar was in question. In our experience the heating of BG medium to 75° to convert it to 'chocolate' gives growth only slightly inferior to that obtained on the unheated medium. By contrast the growth of *H. influenzae* is greatly enhanced on the heated medium. Since Pollock (1947) emphasized the value cf serum albumin, and Verwey *et al.* (1949) found that washed blood cells promoted growth in a medium which did not support growth without this addition, we examined the different constituents of blood.

Haemolysed blood. Rabbit blood haemolysed by alternate freezing and thawing was equivalent to unhaemolysed blood.

Serum. Good growth was obtained on a medium consisting of potato glycerol agar base to which serum was added. Horse, sheep, human or rabbit sera were satisfactory. The variation in species produced less variation of growth of *H. pertussis* than was obtained with whole blood, although rabbit serum was superior to horse serum. When different concentrations of serum were used the best results were obtained with 15–25 % (v/v); 20 % of serum was, therefore, chosen. In general, rabbit serum was used, and samples from a number of different varieties of rabbit appeared to be identical in effect. An adverse effect on the serum was obtained by heating at 75°, but not at 60°, and was much more pronounced than the effect of heating the complete BG medium at 75°.

Blood corpuscles. Although Verwey *et al.* (1949) reported that the addition of human blood cells to their partly defined medium produced a medium better than BG medium for growth from heavy inocula, we found that washed cells had very little value in promoting growth when used in making BG medium. The same batch of BG base which gave profuse growth with whole blood gave no discernible growth in 3 days when carefully washed red cells were used in equivalent amount. The washed red cells were ineffective whether haemolysed or not. Growth in serum agar was augmented by adding a solution of haemolysed washed red blood corpuscles, the increase being proportional to the amount added.

Effect of different samples of human blood. Four samples of blood from students who had had attacks of whooping-cough and four others from students with no history of whooping-cough were compared in BG medium; growth was good and identical with all samples.

It appears therefore, that the blood serum is a specially important component in BG medium; both large- and small-molecular constituents appear to be necessary since ultrafiltration of serum yields two relatively inactive parts (filtrate and residue) which, if recombined, promote growth almost as much as the unfractionated serum.

Peptone and amino-acids. It has already been stressed that peptone was not included in the original BG medium. Nevertheless, since it is often added, the effect of different brands of peptone in BG base was examined. Each of nine different commercial peptones was incorporated (at 1 % w/v) in potato extract glycerol agar base and after the addition of 30 % (v/v) rabbit blood, plates were poured. On inoculation with two recently-isolated strains of *H. pertussis* in heavier and lighter concentrations the results recorded in Table 1 were obtained. Similar results were obtained whether the basal medium was adjusted to pH 7·6 or left without adjustment of reaction as in the original formula of Bordet & Gengou. Table 1 shows a better result without peptone than with any of those added, and that Witte and Difco proteose peptones were less inhibitory than the others. Examination of these peptones showed that the Witte and Difco proteose peptones were much less hydrolysed than the others.

O'Meara & Macsween (1937) and Balch (1942) drew attention to the varying copper content of peptones and the inhibitory effect of traces of this element on many delicate bacteria. We examined the sensitivity of *H. pertussis* to copper (as copper sulphate) in the BG medium. No inhibition was observed even with amounts of copper up to 2 μg./ml. It would appear, therefore, that *H. pertussis* behaved on our medium like those Gram-negative bacteria which were observed by O'Meara & Macsween.

In view of the possibility that the amino-acid content of the potato dialysate was important, other material containing amino-acids was examined. Since the average total N and amino-N content of peptone is about 12 and 2 % respectively of the dry weight, a series of plates was made up in which casein hydrolysate was incorporated with BG medium in various concentrations about this range of nitrogen content. The product known as Casamino acids (Difco) was at first used. In concentrations of 0·1 % some enhancement of growth was produced, but at 1 % there was partial inhibition and at or about 2 % complete inhibition. A series of plates was prepared in which separate amino-acids were added in the proportions in which they were calculated to occur in 0·1, 1 and 2 % of casein hydrolysate. The results of this series of experiments were not always consistent, but certain effects emerged. Aspartic and glutamic acids tended to promote and serine tended to inhibit growth. Both effects could usually be demonstrated in concentrations of amino-acid of *c.* 0·05 %. The stimulant effect of aspartic acid was found to vary with the preparation used; a recent sample from our usual source of supply used in experiments not here recorded was darker than the original and was much inferior. The presence or absence of impurities in the available preparations of this amino-acid must, therefore, be taken into account by anyone who uses this substance.

Table 1. *Inhibition of growth of* Haemophilus pertussis *by various peptones*

BG basal medium throughout. Different peptones (1 % w/v) were added. Inocula: loopfuls from 1/100 and 1/1000 dilutions of faintly turbid suspension of *H. pertussis*.

	Strain I		Strain II	
Addition	**Heavy**	**Light**	**Heavy**	**Light**
None (control)	+++	++	+++	++
Witte peptone	++	+	++	+
Difco proteose peptone	+	+	+	+
Oxo peptone	3 colonies	–	–	–
Parke Davis peptone	1 colony	–	–	–
Evans peptone	1 colony	–	–	–
Bacto-Difco peptone	1 colony	–	–	–
Difco tryptose peptone	1 colony	–	–	–
Vaillant peptone	3 colonies	–	–	–
Eupeptone	2 colonies	–	–	–

+, ++, +++ = numerous discrete colonies, partly confluent, confluent growth.

Substitutes for potato base in BG medium

Clearly the inhibitory activity of some peptones might explain the failure of blood nutrient agar to support growth. It was, therefore, of interest to observe the effect of excluding peptone from that medium. Meat extract was prepared by simmering minced beef with twice its weight of water for 2 hr. in a glass vessel, and then filtering through lint and filter-paper. The extract was adjusted to pH 7·4, 2 % agar added, the pH adjusted again and defibrinated rabbit blood (30 % v/v) added. This medium, although often inferior to BG medium, occasionally yielded better growths. When oxalated horse blood was used in place of rabbit blood a considerable improvement resulted and growth was obtained very similar in amount to that on BG medium and superior to BG medium prepared with horse blood.

In this meat extract horse blood medium the effect of additions of amino-acids and peptones was similar to and often more marked than that in BG medium. With aspartic acid an effect of some interest was observed. Plates of BG medium + 0·05 % aspartic acid were inoculated not only with strains of *H. pertussis* but also with *H. influenzae*, and although in general the action of amino-acids on the growth of *H. influenzae* was not inhibitory, aspartic acid checked its growth distinctly. Incorporation of this amino-acid in a medium for isolation of *H. pertussis* would presumably have the two-fold advantage of somewhat promoting growth of *H. pertussis* and inhibiting that of *H. influenzae*. In addition, horse blood instead of rabbit blood exerted a further inhibitory action on some strains of *H. influenzae*. These effects are potentially valuable in bacteriological investigation of suspected cases of pertussis, since the swabs received are often heavily infected with *H. influenzae*.

In some instances the incorporation of Difco proteose peptone (1 %) in meat extract horse blood medium resulted in complete inhibition of *H. pertussis* while there was a particularly good growth of *H. influenzae*. The effect on the growth of *H. pertussis* of heating this meat extract medium at 75° was not marked; some slight deterioration was produced which disappeared when fresh serum was added. The growth of *H. influenzae* was, however, much greater than in the unheated plates. Hence it is obvious that the failure of recently isolated strains of *H. pertussis* to grow on heated blood agar is not due to a sensitivity to haematin compounds such as that shown by some strains of *C. diphtheriae* (Glass, 1939*a*, *b*).

We are indebted to the Medical Research Council for a contribution towards expenses and for a personal grant to one of us, and also to the students who co-operated in providing samples of blood. Dr D. E. Nicholson was working as an I.C.I. Research Fellow while engaged on this investigation.

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The Assay of Vitamin B_{12}

I. Factors affecting the Response of *Lactobacillus lactis* Dorner ATCC 8000 to Vitamin B_{12}

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SUMMARY: For maximal zones of 'exhibition' in the plate test for vitamin B_{12} with *Lactobacillus lactis* Dorner ATCC 8000, using the media described, the presence of both oxygen and vitamin B_{12} is necessary. Under anaerobic conditions the vitamin is not an essential growth factor and under highly aerobic conditions growth does not occur even in its presence. Under the conditions of maintenance used variant strains were not detected.

When grown in the presence of vitamin B_{12} the bacteria may store sufficient of the vitamin to render measurements of growth useless for assay purposes: when grown under anaerobic conditions the culture obtained is unsuitable for use as an inoculum. A technique for preparing satisfactory inocula of the test organisms and the conditions necessary for well-defined responses to vitamin B_{12} are described.

A cup-plate test for the assay of vitamin B_{12} has been described by Cuthbertson (1949), Foster, Lally & Woodruff (1949) and Cuthbertson, Lloyd & Pegler (1951). In this method a suitable agar medium deficient in the factor under investigation is inoculated with the test organism and is poured into Petri dishes; cups cut out of the agar are filled with appropriate dilutions of standard or test solutions. On incubation under suitable conditions, growth of the organism may occur in sharply-defined circular 'zones of exhibition' around the cups. The diameters of these zones were found to be related to the concentration of the added growth factor and may be used to compare the amounts of growth factor present in the test and standard solutions. The method for estimating vitamin B_{12} is similar to that for aneurin (Bacharach & Cuthbertson, 1948). In developing working details of the assay, a number of observations were made on factors affecting the growth of *Lactobacillus lactis* Dorner ATCC 8000 and its response to vitamin B_{12}; they are reported in this paper.

Media used

In preliminary work we used a charcoal-treated digest of vitamin-free casein to prepare medium A of the composition shown in Table 1, similar to that of Roberts & Snell (1946). With some batches of medium A the addition of tomato juice was not necessary, whereas with others tomato juice supplied an essential growth factor. We also observed that only a few batches of vitamin-free casein could be used to prepare satisfactory media, but suitable digests could usually be prepared with casein freshly precipitated from skim milk or from reconstituted separated full-cream milk powder. To such digests the addition of tomato juice was unnecessary. Eventually charcoal-treated tryptic digests of reconstituted spray-dried separated milk, or of ether-extracted roller-dried full-cream milk, were found to be as satisfactory as any casein digest tried. Medium B, Table 1, was consequently used in most of this work.

Effect of reducing agents

When tryptic digests of casein or milk were stored under toluene at 4° they deteriorated, becoming useless for assay after 1–3 weeks; that is to say, media prepared from these aged digests gave poor zones of growth with pure vitamin B_{12}. In some instances no growth occurred at all, even after the addition of vitamin B_{12}, but growth zones could be observed with certain crude materials containing vitamin B_{12} and other substances. From this it appeared that a growth factor essential present in the fresh casein digest disappeared under our conditions of storage. An attempt was made to discover sources of this growth factor by a technique similar to the auxanographic method of Pontecorvo (1949). An aged digest was used to prepare a batch of medium A (Table 1). Solutions of vitamin B_{12} and substances under investigation were placed in closely adjacent holes on the plates. No growth occurred around the cup containing vitamin B_{12} unless the solution in the adjacent cup contained the growth factor missing from this aged digest. In this way urine, gastric juice, plasma and certain yeast extracts were shown to contain the missing growth factor, but none of these substances was found suitable for use in the basal medium without further purification, because they contained other substances that interfered with the test. The type of results obtained is shown in Fig. 1. When thiolacetic acid or ascorbic acid was placed in a cup adjacent to a cup containing vitamin B_{12} solution there was a clearly marked zone of bacterial growth in the region into which both the vitamin B_{12} and the thiolacetic or ascorbic acid had diffused.

Table 2. *Composition of vitamin* B_{12}*-deficient agar media*

	A	B		A	B
Casein digest (ml.)*	330	—	Adenine (mg.)	10	10
Milk digest (ml.)†	—	330	Guanine (mg.)	10	10
Glucose (g.)	20	20	Uracil (μg.)	10	10
L-Cystine (g.)	0·1	0·1	Lactose (g.)	2	—
Aneurin HCl (μg.)	500	500	Tomato juice (ml.).	50	50
Biotin (μg.)	4	4	Tween (g.)	0·3	0·3
Folic acid (μg.)	2	2	Salt solution C (ml.)‡	20	20
p-Aminobenzoic acid (μg.)	100	100	Ascorbic acid (mg.)	—	1000
Ca D-pantothenate (μg.)	500	500	K_2HPO_4 (g.)	0·5	0·5
Pyridoxin HCl (μg.)	500	500	KH_2PO_4	0·5	0·5
Pyridoxal (μg.)	—	400	Water to (ml.)	1000	1000
Pyridoxamine (μg.)	—	400	pH	6·8	6·8
Nicotinic acid (μg.)	500	500	Agar (N.Z.) (g.)§	15	18
Riboflavin (μg.)	500	500			

* Prepared according to Roberts & Snell (1946).

† Prepared as follows: make up 240 g. spray-dried skim milk (or 350 g. ether-extracted roller-dried full-cream milk), with 2 l. water; add 600 mg. pancreatin (Morson) and 16 g. $NaHCO_3$ and a layer of ether and toluene and digest 3 days at 37°. Add 7–10 ml. glacial acetic acid, steam 30 min., add 70 ml. glacial acetic acid and treat with 40 g. charcoal (Sutcliffe, Speakman No. 5) for 30 min. Repeat charcoal treatment. All filtrations may be assisted by use of adequate amounts of kieselguhr.

‡ Salts C: $MgSO_4.7H_2O$, 10 g.; NaCl, 0·5 g.; $FeSO_4.7H_2O$, 0·5 g.; $MnSO_4.4H_2O$, 2·0 g.; Water to 250 ml.

§ From Davis Gelatine Ltd., 29–31 Mitre Street, London E.C. 3.

In subsequent experiments with different concentrations of ascorbic acid or thiolacetic acid 1 mg./ml. allowed the aged media to support growth of *L. lactis* in the presence of vitamin B_{12}. Ascorbic acid was consequently incorporated in the medium, 1 mg./ml., and the addition of tomato juice was then found unnecessary.

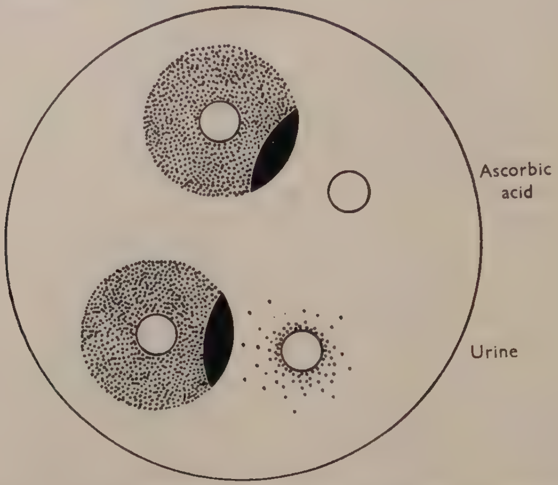

Fig. 1. The effect of reducing agents and urine on response of *Lb. lactis* to vitamin B_{12}.

Effect of thickness of the agar in the plates

A large plate, 15 × 15 in. was poured with 350 ml. of the inoculated agar medium containing ascorbic acid. During the preparation the plate was slightly tilted so that the thickness of the agar varied from 1 to 3 mm. over the breadth of the plate. A solution of vitamin B_{12} was placed at various positions on the plate, which was then incubated overnight. The organism responded well to vitamin B_{12}, showing clearly defined zones whenever the thickness of the agar was between 1 and 2·5 mm.; where the agar thickness was more than 2·5 mm., growth of the bacteria was unrestricted even in the absence of vitamin B_{12}. This effect could be readily repeated in other plates of a similar type. Further, in test-tubes of liquid media containing 0·1 % thiolacetic or ascorbic acid the same unrestricted growth occurred even in the absence of vitamin B_{12}. In other plates in which the agar thickness was less than 1 mm. the organism responded little or not at all to vitamin B_{12}.

Failure to isolate variant strains

During the development of the cup-plate method we frequently encountered difficulty with the assay and often suspected that the properties of the strain had changed, especially in view of the report by Shorb & Briggs (1948) that *L. lactis* very readily produces variants. We were unable at any time to demonstrate differences between the culture in use (maintained by weekly transfer in soya-bean medium) and cultures obtained from freeze-dried samples of the organism. Attempts were made to isolate different strains. On pouring or streaking plates surface colonies were not formed under ordinary aerobic conditions; anaerobically, however, there was good surface growth and colonies could be isolated. No evidence of variation was obtained and we are satisfied that under our conditions of culture this strain is adequately stable.

Effect of different methods of preparing the inoculum

Large inocula are required for this technique. When the organism is grown on a medium containing excessive amounts of vitamin B_{12} ($>0{\cdot}05$ μg./ml.) growth is prolific, but the inoculum thus prepared is useless for assay purposes, as growth occurs even in the absence of vitamin B_{12}, presumably because it is stored in the organisms. This difficulty may be overcome as follows. The organism is grown for 16 hr. in a vitamin B_{12}-rich medium (10 ml. medium without agar or ascorbic acid but with $>0{\cdot}05$ μg. vitamin B_{12}/ml.). The dense overnight growth is washed with saline and resuspended in saline, and 0·2–0·5 ml. of this suspension is used to inoculate 10 ml. of a similar liquid medium free from vitamin B_{12}. On further incubation very great growth occurs; after 5–8 hr. there is a dense suspension, which may be used for assay purposes. Probably the vitamin B_{12} stored in the parent organisms is used up or distributed amongst daughter cells during this phase of very rapid growth on the deficient medium, thus preventing further growth on the plates in the absence of vitamin B_{12}. A technique of this type may also be used in the preparation of inocula of *L. casei* and *L. fermentum* P36 for use in plate assays.

The observation that *L. lactis* does not require vitamin B_{12} in the presence of large amounts of ascorbic acid or thiolacetic acid suggested that suitable inocula might be grown in the absence of vitamin B_{12} but in the presence of these reducing agents. However, such inocula grow prolifically on the plates even in absence of vitamin B_{12}. This phenomenon is being investigated; it appears to be due to the synthesis, under anaerobic conditions, of a substance capable of replacing vitamin B_{12}.

After consideration of the different methods of preparing the inoculum, the one described first in this section was adopted because it was most suitable for laboratory routine. The concentration of vitamin B_{12} used must fall within the limits given; insufficient growth is obtained with less; with more, growth occurs on the test plates even in its absence.

Interference

As stated by Cuthbertson, Lloyd & Pegler (1951), the response of *L. lactis* to vitamin B_{12} is affected by inhibitors of bacterial growth and by deoxyribosides; the former prevent growth and the latter at concentrations of 5 μg./ml. or more cause the zone of exhibition produced with vitamin B_{12} to become diffuse, thus interfering with the assay.

The conditions essential for well-defined response of Lactobacillus lactis *to vitamin B_{12}*

(1) The medium must be aerobic; (2) reducing agents must be present; (3) excess of oxygen must be avoided (excess oxygen may exert its effect by removing the reducing agent); (4) the dilutions put in the cups must not contain more than 5 μg. deoxyribosides/ml.; (5) antibiotics must be absent.

From our results it appears that under highly reducing conditions (or in anaerobic media) vitamin B_{12} is not required for growth. These conclusions are also consistent with the observations of Greene, Brook & McCormack (1949) and Koditschek, Hendlin & Woodruff (1949). Further work should show whether this is because under such conditions the vitamin B_{12} is synthesized, replaced by another substance that can only be formed anaerobically or concerned only in reactions, such as those involving oxygen or H_2O_2, that are not involved in these circumstances.

In the presence of oxygen vitamin B_{12} is required, but alone it is insufficient, for a growth factor present in some tryptic casein digests or in tryptic milk digests is also needed. This growth factor is readily destroyed on exposure to oxygen, but is restored to activity or preserved from destruction by 0·1 % thiolacetic acid or 0·1 % ascorbic acid.

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Relationships between Cell Growth, Surface Properties and Nucleic Acid Production in Normal and Penicillin-Treated *Micrococcus pyogenes*

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SUMMARY: The time course of the changes in the percentages by weight of ribonucleic acid, deoxyribonucleic acid and nucleic acid fragment (%RNA, %DNA and %Nt respectively) and of their absolute rates of synthesis in a strain of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) has been studied throughout a complete normal growth cycle and compared with the changes which occur when penicillin G is added to the culture in the late logarithmic phase of growth. In normal cultures, the rate of cell growth (as dry weight) appears to be controlled by the %RNA, while the %Nt varies inversely with the rate of synthesis of RNA, the Nt/(RNA + DNA) weight ratio being poised at about 0·1 during rapid growth. The rate of synthesis of DNA and the %DNA, on the other hand, are related to the size of the cells in such a way as to maintain the weight of DNA per cell constant. The presence of 1 μg. of penicillin/ml. of culture at a cell dry-weight concentration of 200 μg./ml. causes an accumulation of free nucleotide + nucleoside and a depletion of free purine, and a change of the Nt/(RNA + DNA) ratio from *c.* 0·1 to *c.* 0·2. The absolute rate of RNA synthesis is decreased more than that of DNA, but initially both %RNA and %DNA increase because of the inhibition of synthesis of some unidentified component which contributes significantly to the cell dry weight. The synthesis of an unidentified phosphate ester (XP) is also inhibited at an early stage in the penicillin-treated cultures. At the same time as the chemical changes, there are changes in the surfaces of the penicillin-treated cells, shown by their light-scattering properties and suspension stability. An attempt has been made to integrate the new observations into the general picture of penicillin action.

In the search for an explanation of the mode of action of penicillin upon sensitive organisms there has been a tendency to seek for a key reaction which may be considered to be the cause of toxicity. This attitude presupposes that the pathological symptoms exhibited by penicillin-treated cells represent the later stages in a sequence of events initiated by the key reaction. If the initiating step were known, the subsequent changes might be deduced as the dependent systems were discovered; but we are, in the reverse situation, attempting to guess the nature of the initiating reaction from the comparatively gross changes which follow it. Much is to be gained by a knowledge of the initiating step, but there is some danger that the importance of this step as the key to the subsequent changes may obscure the fact that each event leading from the initial contact with penicillin to the final loss of viability of the organism is equally instrumental in bringing about the lethal effect and is consequently of equal relevance to the description of the toxic process. The likelihood of success in identifying the salient features of the toxic process would seem to depend upon the study of a broad range of physiological indices, and upon our ability to trace back the observed changes to the earliest possible moment.

The observations of Cooper & Rowley (1949) and of Maass & Johnson (1949) on the uptake of isotope-tagged penicillin by penicillin-sensitive organisms indicate that the first of the sequence of events is the spontaneous combination of the penicillin with one or more cell components. If the evidence of Krampitz & Werkman (1947) and of Gros & Machboeuf (1948) for the inhibitory effect of high concentrations of penicillin on thick washed-cell suspensions is applicable to more dilute systems, one of the cell components with which the penicillin combines is presumably associated with the normal functioning of the nucleotide dissimilation systems. In resting or respiring non-growing cells the disturbances caused are comparatively slight and do not set in motion the changes leading to the lethal effects. When growth occurs in the presence of penicillin, however, the lethal changes occur in proportion to the amount of growth (Lee, Foley & Epstein, 1944).

One consequence of growth in bactericidal concentrations of penicillin is damage to the mechanism responsible for the accumulation of glutamic acid in Gram-positive organisms, and it has been suggested that the starvation of the cells of glutamic acid which follows might be sufficient to account for the bactericidal action (Gale & Taylor, 1947; Gale & Rodwell, 1949): a thesis which, if true, would make the loss of the capacity for accumulating glutamic acid one of the last stages in the lethal action of penicillin. The component with which the penicillin initially combines is unlikely to play any direct part in the operation of the glutamic acid accumulation system, because penicillin does not affect this system in resting cells. There must, at all events, be one or more intermediate steps. It seemed possible that a study of the changes in nucleic acid synthesis in relation to cell growth in normal and penicillin-containing media might yield useful information with which to integrate our view of the course of events. A preliminary report of part of this work has been published (Mitchell, 1949*a*). A more detailed description has also been given (Mitchell, 1950*b*).

METHODS

Cultural conditions

The organism selected for study, the strain Duncan of *M. pyogenes* var. *aureus* (*Staphylococcus aureus*), was grown in a casein digest medium containing 1 % glucose and 0·1 % Marmite. The effect of penicillin (crystalline penicillin G of Glaxo Laboratories) on the growth of the culture was observed at a concentration of 1 μg./ml., equivalent to *c.* 50 μg. of penicillin/mg. cell dry weight. The cultures were incubated at 25° in order to slow the biological processes involved and to allow greater separation of the successive stages in the action of the penicillin than is possible at higher temperatures. The rotated culture method of Mitchell (1949*b*) was employed to give reproducible and favourable growth conditions.

Measurement of dry weight

In the present work, the weight of the cells after three rapid centrifugal washings in distilled water and drying to constant weight at 105° was chosen to represent the total cell material in a given volume of culture, and the rate of dry-weight increase thus determined is described as the cell growth rate.

The technical difficulty of washing a large volume of cells rapidly and without loss was found to be too great to allow accurate measurement of the dry-weight concentrations of the low cell populations in the lag and logarithmic phases of growth, especially in the penicillin-treated cultures (see below). The dry-weight concentration of the cells was therefore estimated turbidimetrically in the culture medium at a wavelength of 700 mμ. with the Beckman Model DU spectrophotometer, using cuvettes giving a light path of 1 cm. and diluting the suspensions appropriately with water to give extinctions not greater than 0·200. The expression $\log_{10} I_0/I = k_\lambda . m/\lambda$ was used to relate the extinction ($\log_{10} I_0/I$) to the dry-weight concentration m in μg./ml. at the wavelength λ in mμ., k_λ being called the scattering coefficient.

During normal growth the value of k_{700} varies within limits of ±10 % about a mean value of 0·90 (Mitchell, 1950*a*). It was anticipated, however, that the pronounced cytological disturbances which occur during growth of *M. pyogenes* in the presence of penicillin might cause a wider variation of k_{700} than that which occurs normally. Turbidimetric estimations of the dry-weight concentration of a penicillin-treated culture were therefore checked by gravimetric determinations of k_{700} on the corresponding samples of cells washed with distilled water, with results similar to those shown in Fig. 8. The value of k_{700} for the penicillin-treated cells rises sharply after a delay of about 30 min., reaching a final value about 70 % above the normal and thus largely invalidating the uncorrected dry-weight determinations. It was therefore considered to be essential to make a gravimetric determination of k_{700} for every turbidimetric dry-weight estimation in the penicillin-treated cultures.

Estimation of nucleic acids and 'free nucleotide'

The percentage by weight of 'total nucleotide' (%NA) was estimated by the direct spectrophotometric method of Mitchell (1950*a*), with the addition of a refinement described later. The total purine-pyrimidine compounds extractable from the cells in cold trichloroacetic acid were determined spectrophotometrically and expressed as a percentage by weight of 'free nucleotide' (%Nt), assuming a specific extinction coefficient of 20 as for the 'total nucleotide'. The percentages by weight of deoxyribonucleic acid (%DNA) and of so-called ribonucleic acid were estimated by an adaptation (Stephenson & Moyle, 1949) of the phosphate method of Schmidt & Thannhauser (1945). The percentages by weight of so-called ribonucleic acid obtained by the above method do not, in fact, represent the weight of ribonucleic acid (RNA) in the cells (Mitchell, 1950*a*; Mitchell & Moyle, 1950). The measurements include a large proportion (often 30 % or more) of phosphate in excess of that belonging to nucleotide units of RNA and they have therefore been called %(RNA+XP). The most reliable RNA values may be obtained from the difference %NA − %(Nt+DNA).

General experimental procedure

Two litres of medium, stirred and aerated at 25° in a rotated 5 l. flask was sown with 100 ml. of inoculum in the stationary phase of growth. Samples were withdrawn, beginning with a volume of 200 ml. and diminishing as the dry-weight concentration of the culture increased so as to keep the dry weight of each sample at about 30 mg. From each sample, 5 ml. was taken immediately for estimation of the extinction at 700 mμ. The remainder was centrifugally washed three times with distilled water without precautions as to loss and made up to a volume of just over 16 ml. Of this, 1 ml. was diluted to 100 ml. for spectrophotometric estimation of %NA (Mitchell, 1950*a*) and estimation of the extinction at 700 mμ.; 5 ml. was dried to constant weight at 105° to give the dry-weight figure for the %DNA and %(RNA + XP) estimations and to obtain the value of k_{700} by which the culture density at the time of withdrawing the sample could be determined; 10 ml. was pipetted into 2 ml. of 30 % trichloroacetic acid and kept at 0° for 30 min. to extract the 'free nucleotide' which was estimated by the extinction at 260 mμ. after diluting 1 : 5 with distilled water. The trichloroacetic acid-extracted cells were washed with distilled water and dissolved in 1 ml. of N sodium hydroxide by incubation at 35° for 15 hr. After neutralizing the solution with 1 ml. of N hydrochloric acid, the DNA was precipitated with 2 ml. of 10 % trichloroacetic acid and separated by centrifuging. The depolymerized RNA + the XP contaminant was estimated by the phosphorus content, using the method of Fiske & Subbarrow (1925), assuming 10·6 mg. to contain 1 mg. of phosphorus. After washing with distilled water, the precipitate of DNA was dissolved in 1 ml. of N sodium hydroxide and estimated by the phosphorus content in the same way as the RNA + XP.

The penicillin was introduced into 2 l. cultures in 1 ml. of sterile medium, and initial samples were taken immediately before and immediately after adding the penicillin.

RESULTS

Typical results of observations on a normal and a penicillin-treated culture (chosen from three pairs of similar experiments the results of which did not differ from each other significantly) are given in Table 1. Reference to the figures for k_{350}/k_{700} shows that in the penicillin-treated culture, the deviation of the scattering spectra from the inverse law would introduce a significant error in the evaluation of %NA if this were done in the normal way (Mitchell, 1950*a*). A simple refinement was consequently introduced to allow for the change of the slope of the scattering spectrum in each cell sample. Following the accepted method of expressing the relationship between wavelength and extinction (e.g. LaMer, 1943), the scattering spectrum from 700 to 260 mμ. is assumed to obey a law of the form $(\log_{10} I_0/I) = k.m/(\lambda)^n$, the symbols having the same meaning as before. It follows that the correct value for the extinction due to scattering at 260 mμ. may be obtained in terms of that at 350 and 700 mμ. from the relationship $(\log_{10} I_0/I)_{260\,\text{sc.}} = (\log_{10} I_0/I)_{350} \times (350/260)^n$, where $2^n = 2k_{350}/k_{700}$. In order to facilitate the determination of $(\log_{10} I_0/I)_{260\,\text{sc.}}$, a table of values of $(350/260)^n$ for the observed range of values of k_{350}/k_{700} was drawn up (Table 2).

To compare the behaviour of the normal and penicillin-treated cultures, time 0 in the culture immediately before the addition of the penicillin is fitted on the time scale of the normal culture by means of the dry-weight concentration. Time 0 in the penicillin-treated culture of Table 1 is thus found to correspond to 5 hr. in the normal culture.

Fig. 1 shows that in the normal culture the %NA varies with the rate of growth, as previously noted (Mitchell, 1950*a*). The presence of penicillin causes a transient rise in the %NA followed by a fall which runs parallel to the fall in the rate of growth.

Table 1. *The time course of the changes in the dry-weight concentration, %NA, %Nt, %DNA, %(RNA+XP),* k_{350} *and* k_{700} *in normal and penicillin-treated cultures of* Micrococcus pyogenes

le .)	ODc 700	DWk	DWg	ODw 700	ODw 350	ODw 260	ODe 260	k_{700}	$\frac{k_{350}}{k_{700}}$	ODs 260	ODa 260	% NA	% Nt	% DNA	% (RNA+XP)
								Normal							
0	68	51	24·0	32	57	152	212	0·93	0·89	74	78	16·3	4·43	2·11	22·5
5	99	72	24·6	34	59	169	161	0·97	0·89	76	93	18·9	3·29	2·38	25·6
5	288	214	41·6	56	105	327	182	0·94	0·94	138	189	22·7	2·18	2·35	30·1
5	642	475	42·2	57	113	334	184	0·94	0·99	152	182	21·6	2·18	2·65	27·9
5	1490	1220	44·4	54	111	337	215	0·85	1·03	152	185	20·9	2·42	3·32	24·6
5	2995	2480	53·0	64	138	388	207	0·84	1·08	192	196	18·5	1·95	3·32	23·8
5	4820	3660	62·2	82	174	449	202	0·92	1·06	241	208	16·7	1·62	—	—
5	5270	3800	54·0	75	158	383	122	0·97	1·05	218	165	15·3	1·13	3·55	20·9
								Penicillin-treated							
·0	240	187	47·6	61	115	360	235	0·90	0·94	152	208	21·9	2·47	2·44	28·4
(1·8 mg. of penicillin G added to give 1 μg./ml. of culture)															
·0	228	179	47·0	60	111	352	227	0·89	0·92	144	208	22·1	2·42	2·32	28·5
·5	280	217	32·6	42	75	258	230	0·90	0·89	97	161	24·7	3·60	2·63	28·2
·5	365	220	37·4	62	108	307	235	1·16	0·87	138	169	22·6	3·14	3·02	25·2
·0	688	365	45·0	85	138	340	267	1·32	0·81	170	170	18·9	2·97	3·01	20·6
·0	508	246	40·2	83	134	302	208	1·44	0·81	165	137	17·1	2·58	3·22	19·5
·0	364	192	28·4	54	89	198	143	1·33	0·82	110	88	15·5	2·51	3·10	18·7

Wk is the dry-weight concentration in μg. cells/ml. of culture calculated from ODc 700, the optical density (1000 × I_0/I) of the culture at 700 mμ., and the value of k_{700}. DWg is the weight in μg. of the cells in 5 ml. of the washed pension dried at 105°. The ODw 700, ODw 350 and ODw 260 values are the optical densities of the washed suspen- diluted at 1:100, at 700, 350 and 260 mμ. ODe 260 is the optical density at 260 mμ. of the Nt of the washed pension diluted 1:5. ODs 260 and ODa 260 are the optical densities at 260 mμ. corresponding to scattering and orption respectively, calculated from ODw 350, ODw 260 and k_{350}/k_{700} with the aid of Table 2. Other symbols as ext.

Table 2. *The dependence of* $(350/260)^n$ *upon* k_{350}/k_{700}

k_{350}/k_{700}	$(350/260)^n$
0·80	1·23
0·85	1·26
0·90	1·29
0·95	1·32
1·00	1·35
1·05	1·38
1·10	1·40
1·15	1·43
1·20	1·46
1·25	1·49

The %Nt of the normal culture, shown in Fig. 2, is high in the late lag phase and falls rapidly as logarithmic growth is established. The high value of %Nt at the time of taking the first sample (time 0) was presumably preceded by a rapid rise during the period of 2·5 hr. which elapsed between putting in the inoculum of cells in the stationary phase of growth and withdrawing the first sample. The addition of the penicillin to the culture causes an immediate and very marked rise in the %Nt followed by a fall which is less pronounced than that which occurs during normal growth (Fig. 2).

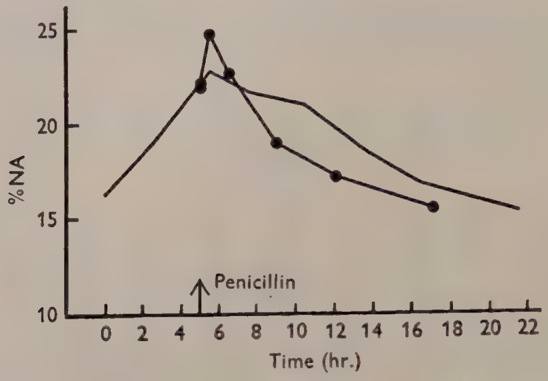

Fig. 1. The percentage by weight of 'total nucleotide' (% NA) versus time in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

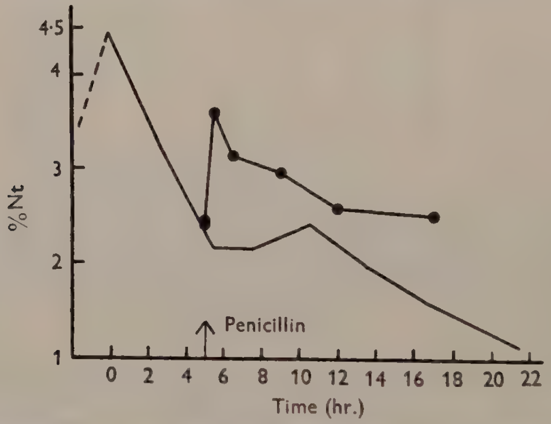

Fig. 2. The percentage by weight of 'free nucleotide' (% Nt) versus time in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

Most of the rise in %NA may be attributed to the rise in %Nt as shown in Fig. 3 by the plot of %(NA−Nt) which is equivalent to %(RNA+DNA).

The balance between the weight of 'free nucleotide' (Nt) and the weight of total nucleic acid (NA−Nt) is plotted in Fig. 4. In the normal culture, the Nt/(NA−Nt) balance favours a high concentration of Nt during the lag phase, but settles down in the logarithmic phase to a value which remains fairly steady throughout the subsequent growth of the culture. The presence of the penicillin causes the balance to swing rapidly in the direction of 'free nucleotide', an almost steady value of Nt/(NA−Nt) being attained after only 30 min. growth in the penicillin-containing medium.

The %DNA, plotted in Fig. 5, rises during most of the growth period during which observations were made on the normal culture. This rise is presumably preceded by an equivalent fall during the early lag phase of growth. The presence of penicillin causes an immediate increase in the %DNA towards the same value as that which is ultimately attained in the normal stationary phase of growth.

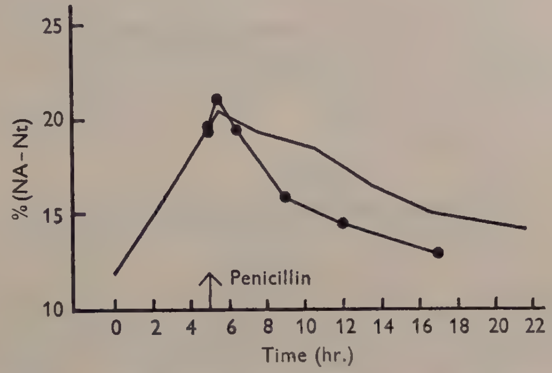

Fig. 3. The percentage by weight of total nucleic acid % (NA – Nt) versus time in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

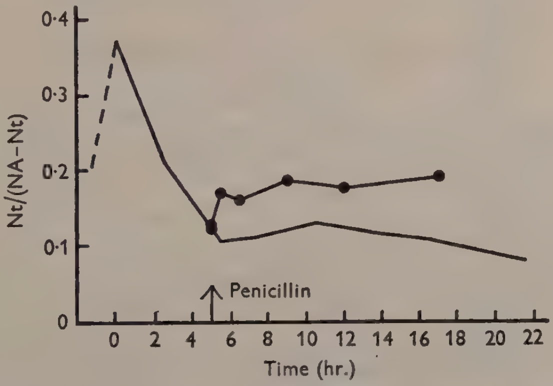

Fig. 4. The weight ratio of 'free nucleotide' to total nucleic acid [Nt/(NA – Nt)] versus time in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

A change in the percentage by weight of a given cell component may be caused either by a change in its rate of synthesis or by a change in the rates of synthesis of other cell components which contribute to the dry weight of the cells. Consequently, the changes in the percentages by weight of the components described above must not be interpreted as necessarily indicating corresponding changes in the rates of synthesis of these components. The changes in the net rates of synthesis are given by the rates of change of the total concentrations in the culture.

Fig. 6 shows the normal cell growth curve and the curves for growth of total nucleic acid and of DNA. During normal growth, in the period preceding the maximum rate of cell growth, the rate of reproduction of total nucleic acid is greater than that of cell dry weight, while in the period following the maximal rate of cell growth, it is less: thus accounting for the maximum in the percentage by weight of total nucleic acid near the centre of the logarithmic phase of growth. On the other hand, the rate of reproduction of DNA is slightly greater than that of cell dry weight throughout most of the growth period: thus accounting for the increase in the %DNA.

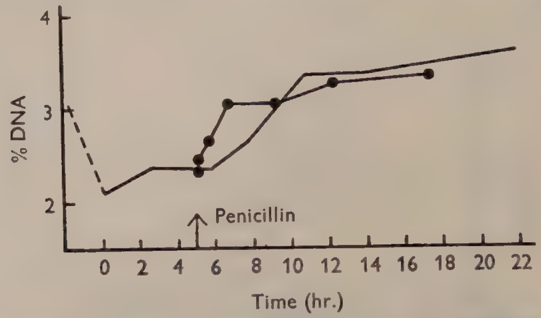

Fig. 5. The percentage by weight of deoxyribonucleic acid (% DNA) versus time in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

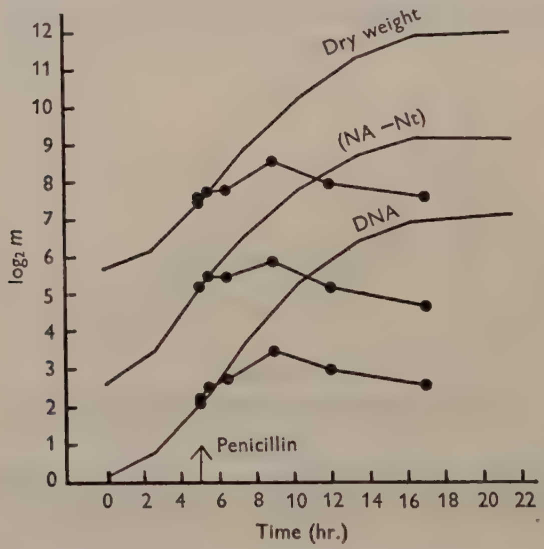

Fig. 6. The rates of reproduction (expressed as $\log_2$ of the concentration in μg./ml.) of cell dry weight, total nucleic acid (NA – Nt) and deoxyribonucleic acid (DNA) in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

After the addition of penicillin to the culture, cell growth is more or less logarithmic for the first 4 hr., but the apparent mean generation time is much extended. During the first 30 min., there is little or no decrease in the rate of synthesis of total nucleic acid, and little or no decrease in the rate of synthesis of DNA, whereas there is a significant fall in the rate of cell dry-weight production. Consequently, the small initial rises in the %(NA – Nt) and %DNA after the addition of the penicillin to the culture may be attributed, not to a stimulation of production of these components, but to an inhibition of synthesis of some other component which contributes significantly to the cell dry weight. After growth has continued for 90 min. in the presence of the penicillin, the cell growth rate is decreased to about 0·2 of its initial value, the net rate of synthesis of total nucleic acid is decreased slightly more, but the net rate of DNA synthesis is decreased only to 0·6 of the initial value: thus accounting for the fall in the percentage by weight of total nucleic acid and the continued rise in the %DNA at this stage.

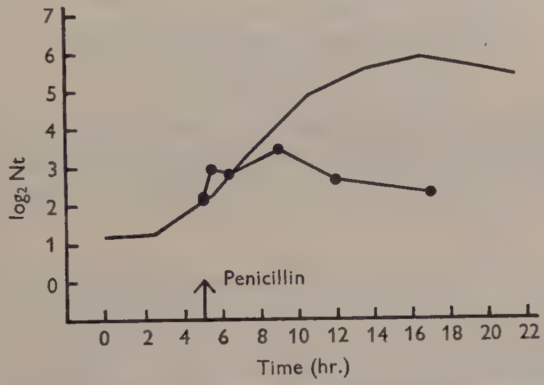

Fig. 7. The rate of reproduction (expressed as $\log_2$ of the concentration in μg./ml.) of 'free nucleotide' (Nt) in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (–●—●–).

The curve of Fig. 7 shows that the growth of Nt in the normal culture is less rapid than that of cell growth during the period of observation, in conformity with the fall in %Nt. It is evident that, unlike the other cases, the rise in %Nt which occurs immediately after the addition of the penicillin to the culture is due to an absolute stimulation of Nt production.

The values of k_{700} of Fig. 8 show that for the first 30 min. after the addition of the penicillin, the value of k_{700} remains unchanged, but it subsequently rises precipitately. The rise in the scattering coefficient of the penicillin-treated cells is an index of changes which increase the proportion of the incident light reflected by the cell surfaces per unit dry weight. These changes might be due either to an extension of the effective reflecting surface area of the cells per unit dry weight, or to an increase in reflecting power of the surface, or to a combination of both types of change. It has been pointed out that the steepness of the scattering spectrum represented by k_{350}/k_{700} is, on the other hand, almost independent of wide changes in the reflecting power of the cell surfaces, but that it is a sensitive index of particle size (Mitchell, 1950*a*). The variation of size during growth of the normal cells and of cells treated with penicillin is illustrated in Fig. 9. The normal cells become progressively smaller throughout the period of growth under observation, a corresponding increase in size presumably occurring during the early lag phase. Microscopic observations on the normal cells showed that the mean diameter changed progressively from about 0·8 μ. in the early log phase to about 0·65 μ. in the stationary phase of growth. Fig. 9 shows that the cells begin to swell immediately after contact with the penicillin. It was noted that the swelling and increase in the scattering coefficient were accompanied by a change in the dispersibility of the washed cell suspensions, the centrifuged pads of cells exhibiting a remarkable 'stickiness'.

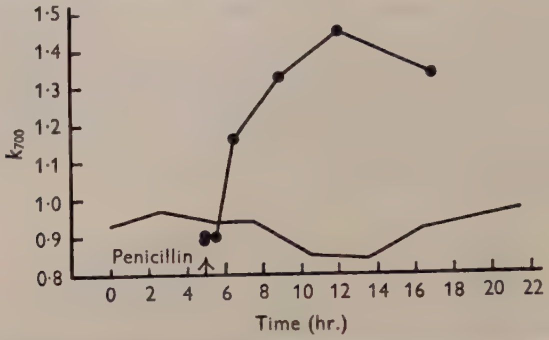

Fig. 8. The light scattering power of the cells given by the index k_{700} versus time in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

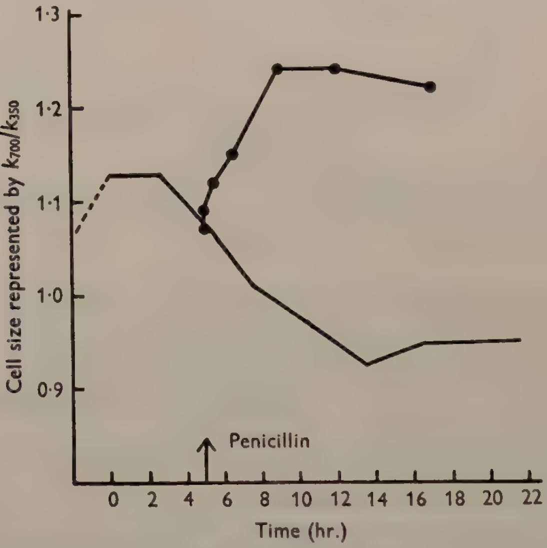

Fig. 9. Cell size versus time in a normal culture (——), and in a culture containing 1 μg. penicillin G/ml. (-●—●-).

In order to examine more closely the changes in the %Nt during the first hour of growth in the presence of the penicillin, an experiment similar to that of Table 1 was devised in which the samples were taken immediately before and after the addition of the penicillin and thereafter at intervals of 10 min. The absorption spectra of the samples were determined in 1 % trichloroacetic acid at the same concentration as that previously used for the determination of the extinction of the Nt fraction at 260 mμ., and the dry weights of the cells were measured directly as before. Fig. 10 shows that the %Nt rises almost linearly from the moment at which the penicillin makes contact with the cells, reaching a value more than 50 % greater than that of the control at the end of the 1 hr. period of the experiment. At the same time there is a movement of the absorption maximum of the Nt towards the red end of the spectrum (Table 3). The movement of the spectrum suggested a change in the balance between free purine and pyrimidine, and the corresponding nucleotide and nucleoside in

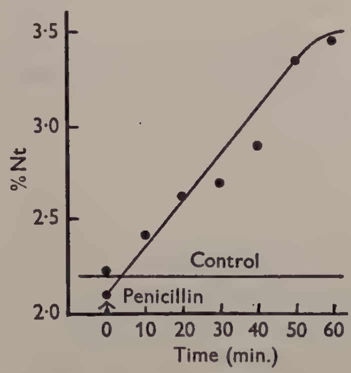

Fig. 10. The early changes in the percentage by weight of 'free nucleotide' (% Nt) in a culture containing 1 μg. penicillin G/ml. (-●—●-) compared with a normal control (——).

Table 3. *The effect of* 1 *μg. penicillin G/ml. on the absorption spectrum of the Nt fraction of growing* Micrococcus pyogenes *var.* aureus

	Time of growth in penicillin-containing medium (min.)							
mμ.	N_0	P_0	10	20	30	40	50	60
250	336	349	376	406	407	436	504	517
255	598	422	464	492	503	535	619	641
258	414	447	475	519	532	571	660	680
260	418	445	483	527	540	581	669	692
262	417	443	483	527	540	584	673	697
265	404	430	472	513	532	576	654	685
270	346	370	409	447	467	507	586	605
275	261	278	309	337	365	388	453	458
280	182	190	210	232	256	274	316	321
290	80	77	86	86	102	105	117	118
300	29	21	22	22	34	26	28	26

The body of the table contains the values of the optical density ($1000 \times \log_2 I_0/I$) in 1 % trichloroacetic acid of the trichloroacetic acid extracts from 1 mg. dry weight of cells/ml. N_0 and P_0 are the samples taken immediately before and after adding the penicillin. Sample P_0 may have continued growing during centrifuging for a maximum of 5 min.

the direction of increasing nucleotide + nucleoside concentration (Hotchkiss, 1948; Ploeser & Loring, 1949). In order to examine this possibility, the Nt fraction of a normal culture of population density corresponding to a dry-weight concentration of 650 μg./ml. and of the same culture after 30 min. growth in the presence of 1 μg. penicillin G/ml. was separated into free purine, total purine and total pyrimidine fractions by the method of Kerr & Seraidarian (1945). The absorption spectra of the fractions were determined. Although the extinction values of the free and total purine fractions from the normal and penicillin-treated cells were different, the shapes of the spectra were identical. It was consequently inferred that the changes of composition of Nt which give rise to the change of absorption spectrum in the penicillin-treated cells is not due to a change in the relative proportions of the purine bases. On the other hand, growth in the penicillin-containing medium for 30 min. caused the total purine to increase by a factor of 1·19, while the free purine decreased by a factor of 0·54: thus showing a substantial accumulation of purine nucleotide + nucleoside. The absorption spectrum of the pyrimidine fraction also indicated a change in conformity with an accumulation of nucleotide + nucleoside in the penicillin-treated cells, the absorption maximum moving from 260 to 264 mμ. It was concluded that both the increase in the extinction and the change in the absorption spectrum of the whole Nt fraction of the penicillin-treated cells could be attributed to an accumulation of purine (and probably also pyrimidine) nucleotide + nucleoside.

In the computation of the net rates of synthesis of the cell components from data such as those of Table 1, it was assumed that the k_{700} value of the cells growing in the culture is unchanged by washing in distilled water. Because of the dubious validity of this assumption, and because there might have been a variable loss of dry weight during the washing operation, particularly in the penicillin-treated cells, it was not certain that the changes in the net rates of synthesis of NA and of the other components, computed from Table 1, were correct. It seemed advisable, therefore, to attempt to measure the %NA and the volume concentration of NA direct in a growing culture so as to avoid the above uncertainties. The very strong ultraviolet light absorption of the medium made direct photometric measurements of NA impossible unless the medium were diluted at least 20 times with water. It was feared that dilution might cause disturbances which would mask the effects which it was intended to reveal; but it was found, on the contrary, that dilution caused a slowing of the toxic process and thus facilitated the observation of its progress.

A culture of *M. pyogenes* was grown in 5 ml. of the normal medium at 35° until the stationary phase was reached. The cells were centrifuged down and resuspended in 5·5 ml. of fresh sterile medium. Two 1 l. conical flasks, each containing 50 ml. of sterile 0·2 % glucose, were inoculated with 2·5 ml. of the suspension and held at 25°. After a lag phase of 2 hr., the NA content of 5 ml. samples was estimated by the direct spectrophotometric method against a control of similarly diluted medium. Immediately after each estimation, the cells of the sample were centrifuged down, and the absorption of the medium was determined against the same control. It was thus possible to obtain the value of the extinction at 350 and at 260 mμ. of the cells and medium. The light scattering at 350 mμ. was used in this instance to assess the dry-weight concentrations of the cultures, assuming $k_{350}=0{\cdot}90$, and the NA content of the cells was estimated assuming $k_{350}/k_{700}=1$. The extinction coefficient of the nucleic acid of both cells and medium at 260 mμ. was assumed to be 20 as before.

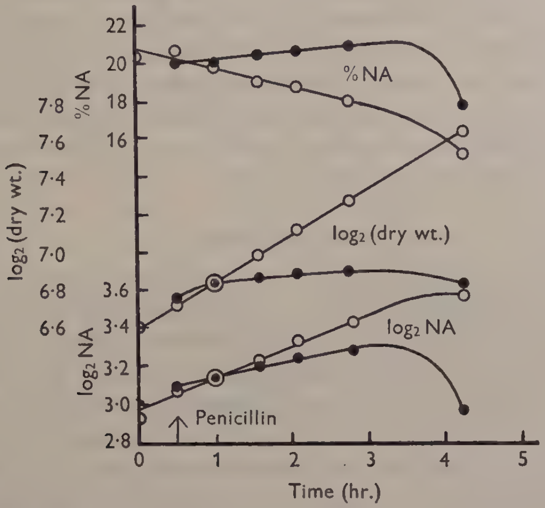

Fig. 11. The rates of reproduction (expressed as $\log_2$ of the concentrations in μg./ml.) of 'total nucleotide' (NA) and cell dry weight and changes in the percentage by weight of 'total nucleotide' (% NA) estimated direct in a normal culture (–○—○–), and in a culture containing 1 μg. penicillin G/ml. (–●—●–).

The point at which the penicillin (50 μg. in 0·1 ml. of distilled water) was put into one of the flasks is indicated in Fig. 11. During the period of growth represented in Fig. 11, the values of the extinction of the medium of both normal and penicillin-treated cultures showed that there was neither accumulation nor loss of purine-pyrimidine compounds in the medium until the time of taking the last sample, when a small increase in the purine-pyrimidine content of the medium was observed in the penicillin-treated culture. The changes in NA content described by Fig. 11 consequently represent purine-pyrimidine compounds synthesized or broken down by the cells, there being no net accumulation from the medium.

For the normal growth, the slope of $\log_2$ NA is constant for more than 2 hr. but less than the slope of $\log_2$ (dry-weight concentration) which is also constant, and hence there is a linear decrease in the % NA over this interval, from the high value of the rapidly growing cells of the inoculum towards a low value commensurate with the slow cell growth rate in the diluted medium.

Since it has been established that k increases with time in penicillin-treated cultures, the slope of $\log_2$ (turbidimetric dry-weight concentration) assuming $k=0{\cdot}90$ must be equal to, or greater than, the slope of $\log_2$ (corrected dry-weight concentration), and similarly, since k_{350}/k_{700} decreases with time in penicillin-treated cultures, the slope of $\log_2$ NA assuming $k_{350}/k_{700}=1$ must be equal to or less than $\log_2$ (corrected NA). Evidently, therefore, in the penicillin-treated culture the rate of cell growth decreases more relative to that of NA reproduction and the %NA rises more steeply than is indicated by the uncorrected values of Fig. 11. The mean generation time is more than 4 hr. for the normal culture in the diluted medium in place of 1·4 hr. in the undiluted medium, and the time taken for the %NA to reach its maximum in the penicillin-treated culture is correspondingly extended by a factor of 3 or more. It is evident that the initial rise in the %NA of the penicillin-treated cultures is due, not to a stimulation of the net rate of purine-pyrimidine production, but to an inhibition of production of some other cell component or components which contribute significantly to the dry weight. The conclusions drawn from the data of Table 1 are thus confirmed.

DISCUSSION

During the growth of normal cultures of *M. pyogenes*, the composition of the cells undergoes wide variation. The range of variation of 'total nucleotide' expressed by the index %NA between a value of about 13 % in cultures in the early lag phase to about 25 % at the period of most rapid growth is approximately the same as that reported by Malmgren & Hedén (1947) on the basis of their microspectrophotometric studies. But, whereas they frequently observed two maxima in the quantity corresponding to %NA in Gram-positive organisms, only one maximum was ever observed in the present work. Unlike the %NA, however, the 'free nucleotide', expressed by the index %Nt, shows two maxima, and it appears significant that both maxima occur during phases of rapid adjustment of the organisms to the nutrient conditions of the medium.

Malmgren & Hedén (1947) made the interesting observation that in a number of bacteria the maximum rate of synthesis of 'total nucleotide' preceded the maximum rate of synthesis of cell substance (as dry weight). They suggested that the hypothesis of Caspersson (1947), that the synthesis of cell protein is preceded by, and is dependent upon, the production of ribonucleic acid, might apply to bacteria as to other organisms. Malmgren & Hedén believed the hypothesis of Caspersson to be confirmed for the Gram-negative organisms which they studied, but there was doubt about the Gram-positive organisms because of the double maxima in the values corresponding to %NA. In the present studies it was noted that the slope of $\log_2$ (NA − Nt) against time reaches its maximum in advance of that of $\log_2$ (dry-weight concentration), thus showing that total nucleic acid is reproduced more rapidly than cell dry weight (the larger proportion of which represents cell protein) during the phase of accelerated cell growth. Subtraction of the DNA from the total nucleic acid given by the index (NA − Nt) shows that it is the RNA synthesis which accelerates most during the phase of accelerated cell growth. The time of maximum percentage by weight of total nucleic acid (or of RNA) follows that of the maximum rate of nucleic acid reproduction by about 1 hr., so that, although the time of maximum rate of cell growth follows that of the maximum rate of nucleic acid synthesis, it occurs almost simultaneously with the period of maximum percentage by weight of nucleic acid. Thus, the rate of cell protein synthesis appears to be controlled by the percentage by weight of nucleic acid in the cells, while the rate of nucleic acid synthesis appears to be more directly determined by the nutritive properties of the medium than protein synthesis—in accordance with the hypothesis of Caspersson (1947).

A similar interpretation may be placed upon the relationship between the percentage by weight of 'free nucleotide' or nucleic acid fragment (%Nt) and that of total nucleic acid. During the lag phase of growth, large amounts of Nt synthesized by the cells or taken up from the medium are accumulated within the cells. As this concentration is built up, the rate of reproduction of nucleic acid accelerates and the %Nt then begins to fall. As the rate of reproduction of total nucleic acid rises towards its maximum, the ratio Nt/(NA − Nt) falls to a fairly steady value near 0·1. Thus, there appears to be a balance between the weights of nucleic acid fragment and total nucleic acid, conditioned by the rate of accumulation of nucleic acid fragment relative to its rate of polymerization to form nucleic acid, the latter being dependent upon the synthesis of the appropriate enzyme proteins.

The observations on the slopes of the scattering spectra described here show the same changes in cell diameter during normal growth as those reported by Henrici (1923). The %Nt runs parallel to the degree of swelling. The %DNA, on the other hand, is 1·7 times as great in the stationary phase of growth as in the late lag phase, while the cell volume changes by a factor of $(0{\cdot}65/0{\cdot}80)^3 = 0{\cdot}54$ during the same period. Assuming the degree of hydration of the cells to remain constant, the weight of DNA per cell must have changed by a factor of only 0·92; or in other words, within the limits of experimental error the weight of DNA per cell remains constant, the amount per cell being about 10^{-15} g.

When a bacterial culture grows without change of composition, the relative net rate of synthesis of each cell component must be proportional to the relative weight of the component in the cells. If all the net rates of synthesis were multiplied by the same factor, no change of composition would result and the survival of the cells would not be endangered. On the other hand, an environmental modification which induces a change in the relative net rate of synthesis of a particular cell component must subsequently lead to a corresponding change of cell composition. This change of composition may either result in the reversible establishment of a new steady condition in which the growth of the cells continues, or it may be such as to render the culture incapable of organized growth. The observations described here show how the changes in the nutritive properties of the medium occurring during normal growth give rise to a wide range of cell compositions between which a reversible movement is possible.

The presence of penicillin in growing cultures of *M. pyogenes* causes the immediate onset of changes in the net composition of the cells. The rapid shift in the ratio of nucleic acid fragment to total nucleic acid [Nt/(NA − Nt)] from its normal value near 0·1 to a new steady value near 0·2 is in conformity with the observations of Krampitz & Werkman (1947) and of Gros & Machboeuf (1948, 1949) on the inhibitory effect of penicillin on nucleotide dissimilation in washed cell suspensions. Since the increase in Nt/(NA − Nt) is accompanied by a slight inhibition of total nucleic acid synthesis and a decrease in the free purine concentration, there is evidently not only a decrease in the net rate of conversion of nucleic acid fragments to nucleic acid but also a net inhibition of free purine production or accumulation.

The rapid initial rise in %DNA, due to the relatively small inhibition of DNA synthesis, confirms the cytological observations of Tulasne & Vendrely (1948) on the inhibition of the growth of a micrococcus in penicillin-containing medium and corroborates their view that 'penicillin interferes with multiplication by inhibition of cytoplasmic rather than nucleal divisions'.

The hypothesis, published by Gale (1948), that penicillin causes loss of the Gram-complex and inactivation of the glutamic acid accumulation system by inhibition of RNA synthesis is proved to be at least partially incorrect for the following reasons: (i) although there is an absolute inhibition of both RNA and DNA production in the penicillin-treated cultures, the percentages by weight of these components initially increase because there is a more pronounced inhibition of some unidentified cell component or components; (ii) doubt has been cast upon the validity of the magnesium ribonucleate Gram-complex theory of Henry & Stacey (1946), by Boivin (1948), and by Mitchell & Moyle (1950); (iii) the intensity of the Gram-staining of the micrococci (Dufrenoy & Pratt, 1947) and their ability to accumulate glutamic acid (Gale & Taylor, 1947) decrease fairly rapidly as growth proceeds in the presence of penicillin, although the level of the %RNA does not fall below that of older cells in which Gram-staining and glutamic acid accumulation are normal. According to Gale (1948), the ratio of DNA to RNA undergoes a large increase in the penicillin-treated cells of *M. pyogenes*. This ratio does undergo a small initial increase because the inhibition of DNA synthesis is somewhat less than that of RNA; but it will be noted that the decrease in the true DNA/RNA ratio is much less than that in the ratio DNA/(RNA+XP), erroneously taken to represent DNA/RNA by Gale. The difference %[RNA+XP]−[%NA−%(Nt+DNA)], which gives an approximate index of the proportion by weight of the component XP responsible for the excess phosphorus of the RNA fraction, drops rapidly in the penicillin-treated cultures (Table 1). Detailed studies on the XP component are in preparation for publication.

The changes in the shapes of the scattering spectra of the micrococci growing in the presence of penicillin are in agreement with the observations of Gardner (1940) and of Duguid (1946), who used visible light microscopy, and of Smiles, Welch & Elford (1948), who used ultraviolet light and electron microscopy, of the swelling of the micrococci in the penicillin-containing media. The rate of change of the slope of the scattering spectra shows, however, that the swelling begins immediately after contact of the cells with the penicillin. This swelling accompanies the increase in %Nt of the penicillin-treated cells just as it does in the normal cells. The rapid increase in the light-scattering coefficient and the decrease of suspension stability which follow the swelling of the cells show that substantial changes in the cell surfaces take place at an early stage in the action of penicillin. The inactivation of the glutamic acid accumulation mechanism in Gram-positive organisms may be associated with the changes in the cell surfaces, but the swelling effects which are known to occur in most organisms exposed to lethal concentrations of penicillin, whether or not they accumulate glutamic acid (Duguid, 1946; Dienes, 1949), suggest a more generalized disturbance of the osmotic and mechanical functions of the cell envelopes than that which would only involve amino-acid accumulation.

Following the preliminary description of our observations (Mitchell, 1949*a*), it was shown by McQuillen (1951) that a decrease in the negative ζ potential of the cell surfaces of *M. pyogenes* var. *aureus* (strain Duncan) accompanies the decrease of suspension stability. According to McQuillen, the changes of ζ potential precede the other changes, but the evidence presented here is not in agreement with his view.

The later stages in the changes initiated by penicillin in *M. pyogenes* probably have little to do with the specific action of penicillin, but are simply typical of the autolytic processes which occur when growth has been stopped. The aim of this work has been to trace back the changes to the earliest one that we can identify. Our progress curves show, however, that the early disturbances follow one another with great rapidity, several of them apparently beginning immediately after contact of the penicillin with the cells. It is therefore impossible at present to decide which of the observed changes may be more closely connected with the initial disturbance. The evidence at present available suggests, however, that the disturbance of 'free nucleotide'/nucleic acid balance and of XP production, and the changes in the cell surfaces, all precede the disturbance to the glutamic acid accumulation mechanism in *M. pyogenes.*

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Observations on the Structure of *Bacillus* Spores

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SUMMARY: An internal organization is revealed in resting spores of *Bacillus* spp. by brief treatment at room temperature with N-HNO_3 or, more effectively, a mixture of N/3-HNO_3 (or HCl) with 0·1 % potassium permanganate. Two distinct steps are involved: the first initiates the reaction without visible changes in the spore; the second is an instantaneous process which abolishes the refractility of the spore and allows staining of the contents. These differentiating procedures have no observable effect on vegetative cells, and the spore's response to them is abruptly abolished with the onset of germination. They are ineffective on heat-killed spores and are inhibited, under certain conditions irreversibly, by 0·5–1 % sodium fluoride. Differentiating reagents probably act indirectly by stimulating abnormal chemical activity of the spore.

Three structures, interpreted as the outer spore membrane, nucleus and cytoplasm, can be distinguished in treated spores. Differentiation does not alter the size or the shape of the spore but shrinks the cytoplasm away from the membrane and, in permanganate-containing mixtures, changes the configuration of the nucleus. The extent of these rearrangements varies with the temperature at which differentiation is carried out. The nucleus is on the outer surface of the cytoplasm and connected with it by a short narrow stalk. The nucleus gives a positive Feulgen reaction. Nucleus and cytoplasm of spores differentiated with N/3-HNO_3 or HCl containing 0·1 % of $KMnO_4$ are soluble in phosphate buffers at pH 6·3–7·6 and M/16 sodium acetate at pH 7·4. Nucleus and cytoplasm are insoluble in these solutions after differentiation with N-HNO_3, but the nucleus of spores treated in this way is destroyed by trypsin in phosphate buffer.

The existence of a second, inner, spore membrane, jointly surrounding nucleus and cytoplasm, is postulated. This layer is believed to be the seat of the spore's bright refractility and imperviousness to stains.

The huge spores of *Metabacterium polyspora* react like *Bacillus* spores to differentiating reagents and permit direct observation of the act of transformation from the featureless to the differentiated aspect of the spore.

A curiously organized cell is concealed beneath the smooth, featureless, noncommittal exterior of the bacterial spore. Two kinds of description of the structure of this cell have been given in the last 15 years. Milovidov (1935), Stille (1937), Delaporte (1939–40) and Piekarski (1940) noted a Feulgen-positive rodlet or granule in the periphery of the spore, but remained silent on the configuration of the interior of the cell. It was eventually realized that after acid hydrolysis at 60° the Feulgen-positive element of the cell is visible as a bright, colourless object at the periphery of the normally featureless spore, and that for the general description of the spore Schiff's reagent could, with advantage, be replaced by staining with ordinary dyes. Accounts of spore structure based on this technique—successfully employed in the study of vegetative forms by Piekarski (1937)—have been given by Robinow (1942, 1945), Klieneberger-Nobel (1945), Flewett (1948), Delaporte (1950), and

Bisset (1950). The newer descriptions have confirmed the presence of a chromatinic structure close to the surface of the resting spore and have provided information on the hitherto undescribed cytoplasmic portion of the cell.

In previous papers (Robinow, 1942, 1945) the cytoplasm was described as occupying most of the space within the spore, separate from the peripheral chromatinic body or nucleus, and differentiated into a basophilic Feulgen-negative core, surrounded by a layer of poorly stainable material. New observations, described in the present paper, have led to a revision of this interpretation. The 'core' is now recognized as the whole of the more or less extensively shrunken cytoplasm, and the clear layer surrounding it as the space between the collapsed cytoplasm and the membrane. New information on the structure of the nucleus and its relation to the cytoplasm has also been obtained.

EXPERIMENTAL

Materials

Bacillus cereus and its variants, because of the large size of its spores and the abundance with which they are formed on ordinary media, has been the chief subject of this study. The strain mainly used was identified by the egg-yolk reaction of McGaughey & Chu (1948) and morphologically. It was originally received from Dr T. F. Flewett as a motile, avirulent variant of *B. anthracis*. *B. cereus* var. *mycoides* and '*B. mycoides* 1110', a smooth *cereus* culture described by den Dooren de Jong (1933), were included among the *cereus* strains. Spores of *B. subtilis*, *B. megatherium*, *B. pumilus*, *B. sphaericus* and *B. polymyxa* were also examined.

In cytological studies on spores the simultaneous presence of different developmental stages and of disintegrating vegetative forms increases the difficulties of interpretation, and only cultures in which mature spores readily become predominant are really useful. Most of the *Bacillus* cultures used in the present study attained this condition after 2 or 3 days at 37° on a simple potato extract agar. (The potato extract is prepared by boiling 200 g. of peeled and chipped potatoes for 20 min. in 1 l. of tap water. The decoction is made up to its original volume and filtered through cotton, 1·5 % agar is added and the pH adjusted to 7·6.)

Spores of *Metabacterium polyspora* (Chatton & Pérard, 1913) from the caecum of guinea-pigs, where they were often found in large numbers, have been examined for comparison with *Bacillus* spores.

Methods of differentiation

Thin films of spores from slope cultures were made in distilled water on grease-free cover-slips and allowed to dry in air. The results obtained with living and fixed spores were identical. For this reason fixatives were not used in this study, except when stated in the text.

The new and very simple methods employed in this study are based on the observation that N-HCl exerts its differentiating effect on spores at room temperature (Robinow, 1945). Higher temperatures are not necessary and damage nucleus and cytoplasm. Later, N-HNO_3 was found to be an even more useful agent. Equivalent concentrations of HNO_3 and HCl affect spores differently. N-HCl and N-HNO_3 are active at room temperature, HNO_3 acting considerably faster than HCl, but only HNO_3 is also active at N/3 at this temperature. Sulphuric acid, surprisingly, proved entirely ineffective over a wide range of concentrations at different temperatures, and N-NaOH likewise had no effect.

The method that was most useful was arrived at unexpectedly. It was found that 1 % potassium permanganate in *c.* N/3-HCl (a powerful spore disinfectant according to Kroenig & Paul, 1897) not only killed spores with great rapidity, but, at the same time, caused differentiation, which had not been expected to occur before subsequent treatment with acid. Applied for 5-10 min. at room temperature, N/3-HCl by itself has a negligible, and 1 % permanganate no noticeable, effect on the morphology and viability of the spores examined. The following differentiating methods were used before staining:

Method 1. N-HNO_3 for 10 min. at room temperature.

Method 2. Potassium permanganate (0·1 %) in N/3-HNO_3 for 5-10 min. at room temperature. The reagent was prepared by mixing 2 vol. 0·15 % potassium permanganate with 1 vol. of N acid; it proved fairly stable and retained its activity for many hours.

Method 3. Potassium permanganate (0·1 %) in N/3-HCl. Mixtures of permanganate with HCl are unstable. Spore films were either treated for 10 min. with freshly prepared solution as in method 2, or the mixture was first allowed to 'ripen' for 30 min. and then applied for 5 min.

All three methods give excellent results with *cereus* spores, but method 1 proved more satisfactory than methods 2 and 3 for spores of *B. subtilis* and *B. megatherium*. Treated films were rinsed several times with distilled water and dried for 1–2 min. while standing on a piece of blotting-paper in the 37° incubator. Unless they had been dried, most of the spores became partly detached from the cover-slip and, in wet mounts, exhibited Brownian motion.

Staining was carried out as follows:

(1) Crystal violet (0·01 %) in water was used throughout. The period of staining varied with the pre-treatment: 0·5–1 min. sufficed after permanganate-HCl; 2–3 min. were required after pre-treatments involving nitric acid. Giemsa solution was used occasionally and gave clear and brilliant pictures.

(2) Feulgen test. Unfixed spores were hydrolysed in N-HCl at 60° for 10–12 min. and then treated with Schiff's reagent for several hours. No counterstain was used.

(3) Preparations made by the osmium-HCl-Giemsa method (Robinow, 1942, 1945) were examined for comparison with the results of differentiating methods 1, 2 and 3.

All pre-treatments and staining were carried out in 'Columbia' staining dishes for cover-slips (A. H. Thomas Company, Philadelphia), which were found more convenient than watch-glasses for the simultaneous processing of groups of spore films. All preparations were examined mounted in water, and were sealed with paraffin. For optical equipment see Robinow (1942). A green light-filter, either Zeiss 'trichrome' or Wratten B58, was always used. A red filter, Wratten F29, proved helpful in resolving the structure of nuclei too deeply stained to be transparent in illumination with green light.

RESULTS

Appearance of normal resting spores of Bacillus cereus

Spores of *B. cereus* are shrouded in a tenuous, ballooning envelope which in some strains protrudes considerably over the ends of the cell (Pl. 1, fig. 1). This 'exosporium' (Lewis, 1934) is plainly visible in suitably stained preparations and invariably in electron-micrographs of *cereus* spores (Pl. 3, figs. 17, 18 and 20), and is quite distinct from the spore membrane proper. The spores differ greatly in size, are brightly refractile and have a faint brownish tint. As a rule no detail of the interior is visible in viable resting spores of *B. cereus* and other bacilli, and none is revealed by straightforward staining, or phase-contrast microscopy.

Internal structure of resting spores of Bacillus cereus

None of the three principal differentiating methods used is without disadvantages. However, the picture of spore structure they furnish between them is consistent and plausible. The configuration and response to differentiating procedures of *nucleus*, *cytoplasm* and *spore membrane* will now be discussed separately.

The nucleus

All differentiating methods reveal a chromatinic structure, interpreted as the nucleus, at the periphery of the cell (Pls. 1, 2, 4 and 5). The shape of this body depends on the method used and, to some extent, on the temperature at which differentiation is carried out. Although there is a good deal of overlap between the results of the various treatments they are sufficiently different to warrant separate description.

The nucleus of most spores differentiated by HNO_3 at room temperature appears in side view as a band of chromatin of uneven density which follows the contours of the cell on one side and frequently curves around one of the poles as well (Fig. 1*a*; Pl. 1, figs. 4, 5). The thinnest nuclei and those best fitting into the normal shape of the spore are seen when differentiation is carried out in the refrigerator at 4–6° (Fig. 2). Seen from the surface, the nucleus appears ovoid or circular. Often its shape is not entirely symmetrical and its edge, where it runs across the surface of the cell, is cut off more or less squarely (Pl. 1, fig. 6) or at an angle or is slightly dented.

Mixtures of permanganate with N/3-HNO_3 or N/3-HCl cause the nuclei to become more or less rounded (Pl. 2). In their changed shape the nuclei cannot be accommodated within the normal confines of the spore and project above the cell surface (Fig. 1*b*; Pl. 2, figs. 8, 11*b*, 12*b*, 13*b*). An advantage of this kind of preparation is the clarity with which the nucleus is revealed as a three-dimensional structure. The nuclei appear to possess a shell of dense chromatinic matter surrounding a lighter centre, especially when they are viewed in red light

(Pl. 4, fig. 26). Vesicles, partly flattened spheres, circular and semicircular disks are the predominant shapes, but since most of the chromatin is in the margin, the immediate impression given by these configurations in lightly stained films is that of rings or crescents. Spores which have become detached from the cover-slip in wet mounts and are tumbling over in Brownian movement allow one to catch a glimpse of the same nucleus from different angles. In this way the 'curved rodlet' type of nucleus described in the past, is readily identified as the lateral aspect of the disk or vesicle configuration. The nuclei are compact and extend over not more than one-half to one-third of the length of the spore. Their contours are remarkably smooth (more so than after differentiation by method 1) and sharply defined (Pl. 3, figs. 19, 20).

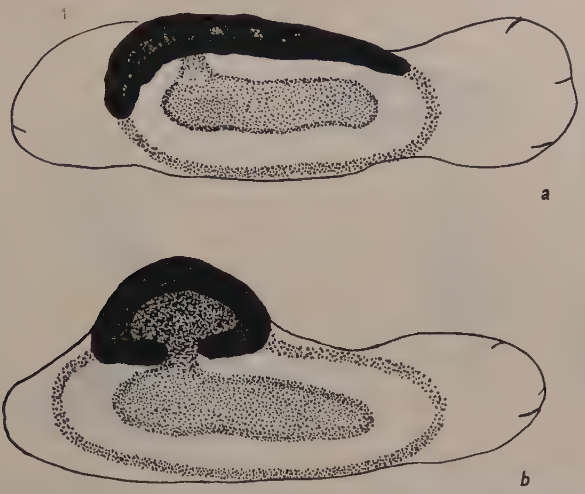

Fig. 1. Diagrammatical longitudinal optical section showing the salient features of spores of *B. cereus* differentiated by method 1, above, and methods 2 or 3 below. The spores are shrouded in a delicate envelope which projects beyond the poles of the cell. The nucleus, the dense structure at the surface of the cell, is connected by a narrow stalk with the central body of shrunken spore cytoplasm. The spore membrane is shown as a stippled band, drawn relatively too wide to conform with the imperfectly resolved image of the membrane in the light microscope. The relationship of the spore membrane to the nucleus, still being a matter of doubt, has been left undetermined.

The nuclei of *cereus* spores differentiated by HNO_3 are destroyed by 25–50 μg. twice-crystallized trypsin (Worthington Biochemical Laboratories, Freehold, New Jersey, U.S.A.)/ml. M/15 phosphate buffer at pH 7·8 in 15 min. at room temperature (Pl. 4, fig. 27), but not by trypsin in water.

No effect on the nucleus was noted after digestion with crystallized pepsin (Armour and Co.) at pH 2·0 in concentrations varying from 30 to 200 μg./ml. at room temperature. Crystalline deoxyribonuclease (Worthington Biochemical Laboratories) weakened, but did not entirely abolish the normally very distinct positive Feulgen reaction of the nuclei. The spores were first hydrolysed at 60° with N-HCl to make the nuclei accessible and then digested in distilled water for 0·5–1·5 hr. with 200–330 μg. enzyme/ml. in presence of a trace of magnesium chloride. The distinctness of the Feulgen reaction of controls incubated in distilled water was not impaired.

The cytoplasm

Regardless of the differentiating method used, part of the interior of the spore is invariably occupied by a centrally placed mass of basophilic matter, cast more or less in the shape of the spore but having a much smaller volume. The central body is more sharply defined in spores treated with permanganate mixtures than in spores differentiated with the aid of plain HNO_3 or HCl. Its size depends markedly on the temperature at which differentiation is carried out. The central mass is relatively large and shows but slight deviation from the proportions of the intact spore when differentiation is carried out in the cold (4–6°) (Figs. 2, 3). Under these conditions when differentiation is by method 3 the central body often appears glossy and is poorly stainable (Fig. 3), indicating that not the spore membrane, but rather the surface of the spore contents is brightly refractile and impervious to stains.

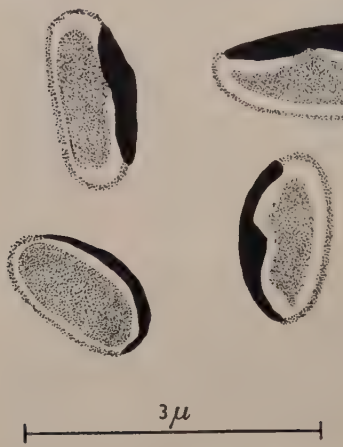

Fig. 2

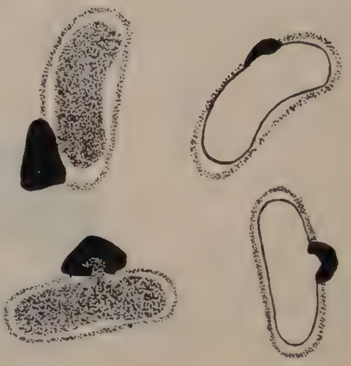

Fig. 3

Fig. 2. Spores of *B. cereus*, from a preparation that had been differentiated for 10 min. with N-HNO_3 at 6° C. The outer spore envelope has been omitted. Explanation in the text.

Fig. 3. Spores of *B. cereus* from a preparation differentiated for 10 min. with N/3-HCl containing 0·1 % $KMnO_4$ at 6° C. The outer spore envelope has been omitted. The central body in the two spores on the right was brightly refractile and only faintly stained. Explanation in the text.

The central body in spores differentiated at room temperature is relatively small and slender (Fig. 1*b*). Somewhere along its side, usually near the middle, there may be continuity between the central body and the nucleus in the shape of a broad neck or short, narrow stalk. This is commonly seen in spores treated by methods 2 or 3; less distinctly or not at all in spores differentiated by method 1. The destruction of the nucleus by trypsin is accompanied by more or less extensive shrinkage of the central body (Pl. 4, fig. 27).

This picture of spore structures, illustrated in Figs. 1 and 2, has formerly been in terpreted by the writer to mean that the cytoplasm of the spore is differentiated into two regions: a basophilic core and a clear poorly stainable outer layer, the whole imagined to be surrounded by a closely fitting and invisible membrane (Robinow, 1945). It now seems more likely that the basophilic core is the remains of the spore's cytoplasm after a process of shrinkage or collapse, and the clear outer region is a free space between the contracted cytoplasm and the more rigid spore membrane.

The new interpretation is partly based on observations on the spores of *Metabacterium polyspora*. *Metabacterium* is an actively motile, peritrichously flagellated organism distinguished by unusual size and the ability to form up to nine spores in one cell. The problem of its cultivation has not yet been solved and its mode of reproduction is uncertain. The length of *Metabacterium* spores varies from 7 to 11μ. Their width is about 2μ (Pl. 1, fig. 7; Pl. 3, fig. 14*a*). Treatment by methods 1, 2 or 3 has the same differentiating effect on these spores as on spores of bacilli, and the interior of the huge cells resembles, on an enlarged scale, the picture of *cereus* spores.

The large size of the *Metabacterium* cells allows the process of differentiation to be followed under the microscope, and enables the observer to form a three-dimensional picture of the shapes that emerge. The transformation of the radially symmetrical, smooth, featureless and brightly shining spores into the oddly shaped transparent structures shown in Pl. 3, figs. 14*b*, 15, has repeatedly been observed: it takes place very suddenly and without warning after the spores have been in contact for several minutes with the differentiating reagent. The spore substance seemed to contract away from the poles of the cell. The configuration of the interior found after treatment with HNO_3 is shown in Pl. 3, figs. 14*b*, 15, and may be described as a hollow disk or domed button arising from the side of a more or less cylindrical, longer part. The latter tapers towards the ends and often shows a distinct constriction opposite the point where, mushroom-like, the peripheral disk arises on a short stalk. Contraction of the contents of *Metabacterium* spores is slight or may be absent when differentiation by methods 1 or 3 is carried out in the cold.

In *Metabacterium* the collapse of the central body can actually be seen taking place. In *Bacillus cereus*, after the same treatment, a similarly shaped body is found, and it seems plausible to assume that there is a similar collapse. The observation that in both species the size of the central body varies with the temperature of the differentiating agent also argues in favour of this view. Lastly, it is supported by evidence from electron-micrographs of differentiated *Bacillus* spores which show a dense core, i.e. the collapsed central body, surrounded, at some distance, by a delicate membrane (Pl. 3, figs. 19, 20; Pl. 6, figs. 32, 33).

In *cereus* spores differentiation has also been followed under the microscope many times, but the small size of the spores and the instantaneous nature of the transformation jointly defeated all attempts to distinguish consecutive steps of the process.

Solubility of nucleus and cytoplasm in phosphate buffer. Experiments with salt solutions, serving as controls to treatments with enzymes, revealed that after differentiation by method 2 the spore nucleus is destroyed and the central body reduced to a mere thread or completely dissolved by M/15 phosphate buffer at pH 6·3–7·6 or by M/16 sodium acetate at pH 7·4. Nuclei and cytoplasm of spores differentiated with N-HNO_3 (method 1) proved insoluble or only slightly soluble in these reagents. This procedure was therefore chosen to prepare spores for digestion with trypsin in phosphate buffer.

Effect of Feulgen hydrolysis on nucleus and cytoplasm. Treatment with N-HCl at 60° now appears more complex than mere hydrolysis. The acid acts first as a differentiating agent and then exerts a hydrolysing effect on the structures which differentiation has rendered accessible. After Feulgen hydrolysis, especially after fixation with osmium tetroxide, the central body is ill-defined and less basophilic than after differentiation at room temperature (Pl. 4, figs. 24, 25). Spores so treated tend to stain evenly all over (cf. Pl. IV, fig. 23 in Robinow, 1945) either because of the accumulation of stainable matter in the space between the much-diminished central body and the spore membrane or because of greater stainability of the membrane itself.

Sometimes the nuclei appear broken up into two or three granules or rodlets or combinations of these (Pl. 4, figs. 24, 25). Arrangements resembling four granules grouped like the 4 on a dice, or three granules arranged in V-shape are common under these conditions in *B. cereus* and *B. cereus* var. *mycoides* where they have been seen by Robinow (1942) and Delaporte (1950). Change of focus reveals in many cases that the rodlets and granules are connected, and it is then easily seen that these clusters are modified ring forms. It is interesting to note that very similar configurations form the chromatinic structures of the vegetative forms of *B. cereus* (Robinow, 1942), where osmium tetroxide causes similar changes in configuration. The loss of a clear view of the cytoplasm caused by Feulgen hydrolysis is thus offset by the demonstration of separate parts in the nucleus, and sharper differentiation of nucleus and cytoplasm than is achieved by more gentle methods of differentiation. It should be remembered, however, that this disjointed aspect of the nucleus is an artefact and that normally its parts are continuous.

A clear-cut Feulgen reaction is given by the basophilic shell of the nucleus (Pl. 4, figs. 21–23). In wet mounts it appears that the central, shrunken body of cytoplasm and the spore membrane also give a positive reaction. However, when the spores are dehydrated with ethanol and embedded in Canada balsam, the magenta colour of the nuclei is intensified, and spore membrane and cytoplasm are no longer visible, in agreement with the results of most of the earlier workers. This suggests that the positive reaction of cytoplasm and spore membrane in water mounts is an optical illusion, due to an unspecific faint reddish tinge, reinforced by the relatively high refractive index of these structures. A final decision is difficult because much of the cytoplasm is lost from the spores in the course of Feulgen hydrolysis.

The spore membrane

One might attribute the striking transformation of the appearance of resting spores, caused by differentiating agents, to drastic changes in the spore coat. But the spore membrane is not impervious to stains, even in the untreated resting spore. This is apparent from the tinted contours and dimmed refractility of spores stained with crystal violet (Pl. 2, fig. 11*a*). Secondly, the spore coat which is discarded during germination is not noticeably affected by the reagents used in differentiation. Thirdly, differentiation by method 3 in the cold causes the separation of contents and membrane, and reveals that refractility and poor affinity for stains are properties of the cytoplasm rather than of the membrane (Fig. 3). These facts point to the interior of the spore as the site of the striking changes wrought by differentiating procedures. Any changes in the spore membrane may simply be the consequence of its separation from the normally impervious, refractile central body, or may be due to an increase in thickness.

So far it has not been possible to decide whether nuclei which protrude above the cell surface merely lift and stretch the spore membrane, or have actually burst through. It is likely that the electron microscopy of differentiated spores whose nuclei have been removed with salt solutions will be helpful in solving this problem. Meanwhile, the ability of the spore membrane to withstand stretching without rupture is evident from the conspicuous humps, caused by underlying rounded nuclei (Pl. 1, figs. 1–3), which arise in the otherwise normal-looking spore surface when certain concentrations of chlorine water or hypochlorite (see below) are used instead of permanganate in differentiation by method 3. Comparison of Pl. 2, fig. 11*a* with fig. 11*b*, when the unnaturally protruding nuclei are disregarded, reveals that size and shape of the volume enclosed by the spore membrane are little changed by differentiation. This implies a certain rigidity of the membrane, at least in the wet state. On the other hand, the drying which is unavoidable in preparing cells for electron microscopy leaves the membrane of *cereus* spores in a much folded and shrivelled condition (Pl. 3, figs. 19, 20).

Structure of the spores of Bacillus subtilis *and* Bacillus megatherium

While *Bacillus* spores differ as regards size and shape of the nucleus and relative size of nucleus and cytoplasm, spores of *B. sphaericus*, *B. polymyxa*, *B. pumilis* and *B. cereus* var. *mycoides* respond to differentiating treatments in very similar fashion. The structure of the spores of *B. megatherium* and *B. subtilis*, in contrast to *cereus* spores, varies little with the temperature and nature of the differentiating agent. Nitric acid (N) at room temperature (method 1) has proved most satisfactory.

B. subtilis. The spores of four strains examined have, in the differentiated condition, a certain neatness and uniformity of appearance. Compact rounded nuclei that project above the cell surface are most commonly seen (Pl. 5, figs. 28, 29; Pl. 6, figs. 32–34). Thin flat nuclei that follow the contours of the spores, recalling those found in *B. cereus* after differentiation with N-HNO_3, are common after very brief treatment or when differentiation has been carried out in the cold. Electron micrographs (which the author owes to the kindness of Dr C. G. Hedén, of the Karolinska Institute, Stockholm) show the membrane remarkably free from folds, the shrunken cytoplasm, and dense angular nuclei. It is tempting to speculate whether a shallow depression in the cytoplasm of some of the spores in Pl. 6, fig. 34, corresponds to the site occupied by the nucleus in the untreated cell before differentiation caused a drastic change in its shape, but it is difficult to judge how much the configuration of the cytoplasm has been influenced by the drying to which cells are unavoidably subjected in electron-microscopy.

B. megatherium. The spores of four strains investigated reacted in the manner of *subtilis* spores, but showed greater variation in cell size and configuration of the nucleus (Pl. 5, figs. 30, 31).

Synopsis of changes induced by differentiating procedures

In reconstructing the sequence of events during differentiation it is helpful to postulate that the site of the spore's bright refractility, its imperviousness to stains and the scene of the first changes in differentiation is not the spore coat but a second, inner membrane surrounding the central body of cytoplasm and the nucleus. (In *Metabacterium* the spore coat often extends a little beyond the glistening spore body at the poles of the cell (Pl. 1, fig. 7), clearly indicating the separateness of the two structures.) The hypothetical membrane is represented by a broken line underneath the spore coat in Fig. 4. Earlier observations on the number of coats in the spore are discussed by Knaysi (1948).

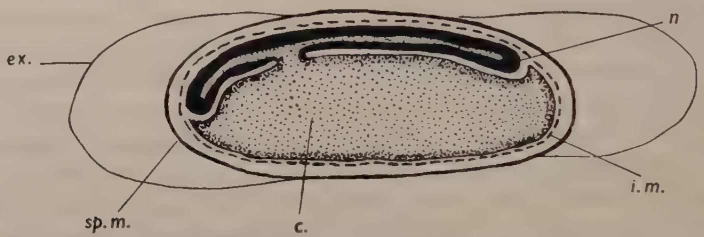

Fig. 4. Diagram of a hypothetical longitudinal section through a resting, untreated spore of *B. cereus*. The delicate 'exosporium' projects over the poles of the cell. Under the spore membrane proper (heavy, continuous line) there is a second membrane (broken line) surrounding both nucleus and cytoplasm. *ex.*, exosporium; *sp.m.*, spore membrane; *i.m.*, inner membrane; *c.*, cytoplasm; *n.*, nucleus.

Three steps in differentiation can be recognized:

(1) Inner and outer spore membranes are jointly lifted but not broken by the rounded nuclei of spores treated with acid mixtures in which chlorine water or hypochlorite have replaced permanganate (Pl. 1, figs. 1–3).

(2) When spores are differentiated by method 3 in the cold, the inner membrane is broken by the protruding nuclei, which consequently become stainable, while the cytoplasm, though shrunken, still retains its refractile and impervious coating (Fig. 3).

(3) Lastly, cytoplasm and nucleus, suffering changes of shape in the process, are both laid open to view and rendered stainable, following the complete breakdown of the inner spore membrane induced by methods 1, 2 or 3 at room temperature (Pl. 2, fig. 8).

The forces which compel the striking changes of shape, and presumably of volume, of the spore nuclei, e.g. in differentiated *subtilis* spores, are not yet understood. It is difficult to resist the impression that the nuclei are under tension in the untreated cell, and are relieved from it when differentiating procedures weaken the constraining power of inner or outer spore membrane, or of both. The seeming contraction or collapse of the spore cytoplasm is probably accompanied by the breakdown of some cytoplasmic materials and the loss of substances through diffusion. Without this assumption it would be difficult to account for the difference between the intense basophilia and turgidity of the nearly mature but still directly stainable spore, and the shrunken moderately basophilic cytoplasm of spores differentiated by methods 1, 2 or 3.

Observations on the nature of the differentiating process

The most efficient differentiating reagents are oxidants. Thus, nitric acid is more effective than hydrochloric acid, and the potency of N/3-HCl, which is almost inert at room temperature, is vastly increased by the addition of permanganate. It was found early in the work that spores killed, i.e. rendered unable to germinate, by flaming or by being boiled in water for 20 min., no longer responded to differentiating treatments. They also fail to show, during subsequent incubation in a nutrient medium, the signs of abortive germination—loss of refractility and increased affinity for stains, followed by early disintegration—which are exhibited by spores killed in many other ways. The ability of spores 'killed' by some methods, to carry out the first few steps of germination indicates the temporary survival of some of the spore's normal metabolic systems. Boiling not only destroys these systems but also the spore's ability to respond to differentiation treatments. This observation raised the possibility that differentiation, like germination, may depend on the functioning of certain entities *inside* the spore, enzymes perhaps, conceived as heat-sensitive and susceptible to activation by certain chemicals.

To test this idea the effect of several enzyme poisons was investigated. It was found that differentiation was inhibited in the presence of approximately 0·1–0·2 M sodium fluoride. In HCl-containing reagents 0·5 % sodium fluoride caused nearly complete inhibition of differentiation and, if allowed to act for 3–5 min., also abolished the response to subsequent treatments with reagents not containing fluoride. A higher concentration of sodium fluoride (1 %) is required to achieve equally complete inhibition of differentiation in N-HNO_3. This effect, in contrast to the action of fluoride in HCl-containing reagents, is largely reversible.

The course of differentiation in 0·1 % potassium permanganate in N/3-HCl was followed quantitatively, with results which are summarized in Fig. 5 and Tables 1 and 2. After it had been allowed to 'ripen' for 30 min. on the bench this mixture caused the differentiation of 80–100 % of *cereus* spores in 4–5 min. In Fig. 5 the proportion of differentiated spores is plotted against time spent in the reagent for four separate experiments. In a fifth experiment most of the spores briefly exposed to the HCl-$KMnO_4$ mixture not only failed, despite thorough washing, to germinate within 7 hr. when covered with heart infusion agar in the incubator, but also showed clear signs of degeneration. The spores in control samples, treated only with permanganate solution, germinated within 1 hr. Although most of the spores are rendered unable to germinate during the first 90 sec. in the reagent, few are visibly changed. That a reaction is started in the spores by the treatment is shown in the high proportion of differentiated spores found when they are first treated for 2 min. in the HCl-$KMnO_4$ mixture, then washed and immersed for another 3 min. in N/3-HCl, a reagent which normally does not affect either the viability or the appearance of *cereus* spores.

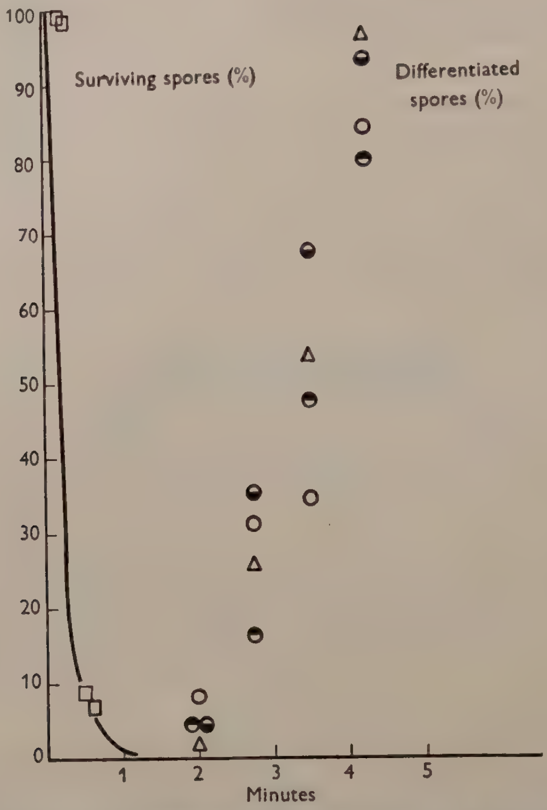

Fig. 5. Reaction of spores of *B. cereus* to N/3-HCl containing 0·1 % potassium permanganate. The curve on the left indicates the decrease in the number of viable spores effected by 45 and 90 sec. treatment with the differentiating reagent. The symbols on the right give the percentage of differentiated spores, in four experiments, as a function of the time spent in the reagent.

Experiments with sodium fluoride present in the reagents during either the preliminary or the final treatment (Tables 1 and 2) have further clarified the course of differentiation and show that fluoride arrests this process in spores in which it has been started by preliminary exposure to a fluoride-free reagent.

It is concluded from these observations that the differentiation of *cereus* spores in N/3-HCl containing 0·1 % permanganate is the result of two distinct reaction steps. The first step initiates differentiation but is not accompanied by visible changes in the structure of the spore; the second step leads to visual differentiation. Both steps are inhibited in the presence of 0·5% sodium fluoride, the second more completely than the first.

The fact that chlorine develops in the mixture of potassium permanganate with HCl suggested that chlorine might be a potent differentiating agent. The effect of water saturated with chlorine gas and of a commercial, approximately 1·5 M solution of sodium hypochlorite ('Javex'; Standard Chemical Company, Toronto) on spores was studied and, provided very low concentrations were employed and the reagents dissolved in 0·33–1·0 N-HCl, differentiation of spores was consistently obtained. One part of chlorine water in 30 parts of N/3-HCl caused 50–80% differentiation in 5 min. at room temperature; higher concentrations were ineffective; without HCl there is no effect. Similarly, 1 vol. of 'Javex' solution + 100 vol. of N/3-HCl caused 20% differentiation in 5 min. Spores when treated with higher than optimal concentrations of the chlorine reagents in N/3-HCl appeared brightly refractile and nearly as impervious to stains as normal spores, though the shape of many of them was no longer symmetrical. A distinct hump (Pl. 1, figs. 1–3) arose on the side of the cell,

Table 1. *Effect of fluoride on the differentiation of* cereus *spores when present during the first* 1·5 *min. of exposure to a differentiating reagent*

(Figures represent percentage of differentiated spores. A total of 2–300 was counted in every preparation in this experiment and the one described in Table 2.)

Treatment	Differentiated spores (%)
(*a*) 1·5 min. in N/3-HCl containing 0·1% potassium permanganate	3·7 2·0
(*b*) Treatment as in (*a*) followed by 3 min. in N/3-HCl	98·5 98·5
(*c*) 1·5 min. in N/3-HCl containing 0·1% $KMnO_4$ and 0·5% NaF	<1 <1
(*d*) Treatment as in (*c*) followed by 3 min. in N/3-HCl	47·0 43·0

Table 2. *Complete inhibition of differentiation when fluoride is present during the last* 3 *min. of the exposure of* cereus *spores to a differentiating reagent*

Treatment	Differentiated spores (%)
(*a*) 1·5 min. in N/3-HCl containing 0·1% $KMnO_4$	22·6
(*b*) Treatment as in (*a*) followed by 3 min. in N/3-HCl	96·0 92·6
(*c*) Treatment as in (*a*), followed by 3 min. in N/3-HCl containing 0·5% NaF	16·5 22·5 15·5
(*d*) 3 min. in N/3-HCl, without pre-treatment	<1 <1

corresponding in size and position to the protruding nucleus of spores differentiated by methods 2 or 3.

The failure of hypochlorite in concentrations above *c.* 0·03 M to cause differentiation, might indicate that the spores are killed by too high a concentration of the reagent before differentiation can begin. If this were true, spores killed with hypochlorite in acid solution should also fail to respond to other differentiating treatments; this was found to be so. Neither by methods 1, 2 or 3 was it possible to induce the differentiation of spores previously treated with 1 vol. 'Javex' + 9 vol. N/3-HCl. But the poisoned spores themselves were able to carry out this reaction. One hour after chlorine-poisoned spores had been incubated in a moist chamber under a slab of heart infusion agar, all had become stainable and showed peripheral nuclei protruding from a strongly basophilic central body of cytoplasm. No further development was observed. The experiment has been repeated several times with the same result.

A somewhat similar behaviour, though not leading to proper differentiation, is shown by spores poisoned by 0·1 M fluoride in mixtures of permanganate with HCl. Although refractory to differentiating treatments, the poisoned spores, when incubated with heart infusion agar, lost their refractility, became diffusely stainable and eventually disintegrated.

These two observations also point to the possibility that the spore's own chemical activity may be an important part of its response to differentiating treatments.

DISCUSSION

The refractile, not directly stainable spores whose structure has been described in the preceding pages represent the normal, heat-resistant and viable resting stage of *Bacillus* cells. Non-refractile and directly stainable 'spores' which have been described from time to time, most recently by Delaporte (1950) for *B. mycoides*, may safely be regarded as the result of premature and incomplete germination. A special type of spore with 'transparent central regions' which Knaysi, Baker & Hillier (1947) have recorded with the electron microscope are found in many *Bacillus* species and have long been known. Examination with the light microscope and slide cultures leave no doubt that these objects are the lifeless remains of autolysed spores.

The structure of the spore cannot be satisfactorily discussed without regard to its development. Knaysi & Baker (1947), relying on the electron microscope, described variously shaped masses of relatively high density in *Bacillus* cells 'grown in a nitrogen-free medium' and have interpreted these alternately as division figures of vegetative nuclei or as developing multi-nucleate spores. In view of the abnormal nature of their material, the unspecific character of the information from electron microscopy and the absence of supplementary documentation based on light-microscopy and conventional cytological methods, it is difficult to assess the significance of these findings.

Cytological aspects of spore formation have lately been described by Klieneberger-Nobel (1945), Flewett (1948), Bisset (1950), and others, who are agreed that the spore arises in the cytoplasm in connexion with one of several nuclear elements present in the sporulating bacillus. As it increases in size the spore becomes increasingly basophilic. Its affinity for stains at this stage differs from that of the cytoplasm of growing bacteria in that it resists hydrolysis with N-HCl at 60°. This makes it difficult to trace the configuration of the nuclear element inside the developing spore. In the most advanced developmental stage that still yields to hydrolysis it has the shape of a ring or ellipse according to Flewett (1948), an observation that needs to be reconciled with Knaysi's statement (1946) that immature spores give a diffuse Feulgen reaction. Flewett believes that the ring gradually expands until it reaches the periphery of the cell.

The peripheral chromatinic structure in *Bacillus* spores is visible unstained, after differentiation, as a well-defined body by the side of the cytoplasm. It has been referred to as the nucleus because its division during germination gives rise to the chromatinic, presumably nuclear, structures of the vegetative cell (Robinow 1942, 1945; Delaporte, 1950). Its affinity for basic stains and its positive Feulgen reaction, as well as its destruction by trypsin and depletion by deoxyribonuclease, are in accord with this interpretation.

The accumulated evidence indicates that at least two distinct structures are contained in the spore: nucleus and cytoplasm; but we are still faced with the problem of their relative arrangement in the untreated cell. Certainty on this point could only be derived from the examination of sections cut from spores, but this has not been attempted. Spores having the structure here described are not without precedent, for peripheral, rod- or disk-shaped nuclei can be demonstrated without recourse to differentiating methods in the spores of a (bacterial?) parasite of some species of *Trichonympha* (Kirby, 1944).

It is obvious that the appearance of *cereus* spores, differentiated with permanganate mixtures at room temperature is an artefact. But the picture of a flat nucleus, moulded to a turgid central body, consistently encountered in films of spores differentiated with N-HNO_3 in the cold, probably comes reasonably close to the natural condition of nucleus and cytoplasm in the spore-cell. In spores differentiated with permanganate + acid mixtures the situation is probably complicated by the nucleus drawing some of the cytoplasm up into itself as it rises above the cell surface (Fig. 1*b*).

The spore loses its refractility and becomes stainable in the course of both *differentiation* and *germination*: phenomena believed to involve a profound change of the properties of the shiny, inner, spore membrane. There ends the similarity between the two processes. When the spore becomes directly stainable in the course of germination the nucleus has already left its peripheral position and has sunk into the cytoplasm (Robinow, 1945). At the same time the germinating cell as a whole no longer reacts with shrinkage and collapse to differentiating reagents (in *B. cereus*, 50 % of the spores are thus transformed within the first 5 min. in heart infusion broth at 37°). In other words, when treated with differentiating reagents, spores in the very earliest stages of germination reveal neither bulging nuclei, nor shrunken central bodies of cytoplasm. It would be interesting to know why it is that resting spores, so resistant to most noxious influences, react violently to reagents which have no visible effect on the structure of the vegetative phase; but the cytological picture does not shed much light on this paradoxical situation.

In the present study the huge spores of *Metabacterium* have served as large-scale models of the condition assumed by differentiated *cereus* spores. This comparison is useful though there is little evidence as yet that the mushroom-like structure in *Metabacterium* spores shares all the properties of its counterpart in *cereus* spores. The results of the Feulgen test have been erratic and germination could not be observed, neither has the study of the formation of *Metabacterium* spores proved helpful in this respect. On the other hand, the similarity in the construction of the spores of the two species is so great that one can hardly ignore it. Gain from the comparison has not been one-sided. The taxonomic position of *Metabacterium* has long been a matter of doubt. By the organization of its spores, its lively motility (overlooked by the first observers) by means of peritrichous flagella and the occasional occurrence of individuals in a state suggesting binary fission, the organism now proclaims itself a true bacterium.

Constant in its organization, easily obtained, handled and processed, the spore provides a useful starting-point for the study of bacterial cytology. Progress in the biology of this enigmatic cell now depends on the chemical characterization of the structures established by morphological studies.

The author is indebted to Prof. R. G. E. Murray for his interest in all phases of the work and many helpful suggestions. He also wishes to thank Dr F. H. Johnson, of the Biology Department, Princeton University, for the hospitality of his laboratory where some of the work was done. Thanks are due to Dr James Hillier of the R.C.A. Laboratories, Princeton, U.S.A., and Dr C. G. Hedén of the Karolinska Institute, Stockholm, for giving of their time and skill in taking electron micrographs of some of the writer's specimens.

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EXPLANATION OF PLATES

PLATE 1

The magnification is the same for all photographs on this plate and is indicated by the scale next to fig. 5.

Figs. 1–3. Spores of *B. cereus* treated for 5 min. with 1 vol. 'Javex' hypochlorite solution in 10 vol. N/3-HCl at room temperature. Stained with crystal violet. A delicate, boat-shaped envelope is protruding over the poles of most of the spores. In some of the spores the nucleus has suffered a change of shape underneath the unbroken spore membrane and is raising a hump in the contours of the cell.

Fig. 4. Spores of *B. cereus* after 20 min. in N-HNO_3 at room temperature. Stained with crystal violet.

Figs. 5, 6. A group of spores from the same preparation that provided fig. 4, photographed at two different levels of focus. In fig. 5 the nucleus of the spore marked with a cross is just visible bending around the left end of the cell. In fig. 6 it is seen from the surface as a dark shield covering the greater part of the cytoplasm.

Fig. 7. One of two spores in an individual of *Metabacterium polyspora* photographed at the same magnification as the other figures on this plate. Compare with fig. 1.

PLATE 2

The magnification of the photographs in this plate is indicated by the scale beneath fig. 8. In the photographs the spore membrane, owing to imperfect optical resolution, appears relatively broad, as if drawn with a soft pencil. In electron-micrographs of *cereus* and *subtilis* spores the membrane is revealed as a very thin structure, lacking the double contours it seems to possess in light-optical pictures.

Figs. 8–10. Spores of *B. cereus* treated for 5 min. at room temperature, with N/3-HNO_3 containing 0·1 % $KMnO_4$. Stained with crystal violet. Note surface aspect of the nucleus in the spore marked with a cross.

Figs. 11*a*, *b*; 12*a*, *b*; and 13*a*, *b*. The (*a*) pictures of the three pairs of photographs represent a group of living, resting spores lightly stained with crystal violet. The (*b*) pictures show the changed appearance of the same group of spores after treatment with N/3-HNO_3, containing 0·1 % potassium permanganate, for 10 min. at room temperature, followed by staining with crystal violet. The protruding nuclei in the (*b*) pictures contrast with the flat, clinging nuclei of fig. 9 on this plate and Pl. 1, fig. 4. Note that the shape of the spores is not altered and their size but slightly decreased by the treatment.

PLATE 3

Magnification of the photographs in the upper half of this picture is the same as that of the figures in the two previous plates and is indicated by a scale beneath fig. 15. The much higher magnification of the electron-micrographs in the lower half of the plate is indicated by the scale underneath fig. 17. These photographs were made by Dr J. Hillier of the R.C.A. Laboratories, Princeton, New Jersey.

Fig. 14*a*, *b*. Two pictures of a pair of *Metabacterium* spores (*a*) before and (*b*) after differentiation with N-HNO_3. Both stained with crystal violet. In the spore on the left the mushroom-like peripheral body which differentiation reveals in these spores is seen from *above*, hence its ellipsoidal appearance.

Fig. 15. Two individuals of *Metabacterium* with two spores in each. Stained with crystal violet after differentiation with HNO_3.

Fig. 16. A group of *cereus* spores, including a few differentiated ones, shown for comparison with the spores of *Metabacterium*.

Figs. 17, 18. Electron-micrographs of resting spores of *B. cereus*. The delicate, much folded, outer envelope of the spores is clearly visible. This envelope is external to the spore membrane proper.

Figs. 19, 20. Electron-micrographs of *cereus* spores, differentiated by method 3, washed and dried. The tenuous outer spore envelope not as clearly visible as in the untreated spores. The spore membrane proper is wrinkled. The nucleus is seen projecting above the cell surface as a dark body with smooth contours. Compare with Fig. 1*b*.

PLATE 4

The magnification of the photographs on this plate is indicated by a scale beneath fig. 23.

Figs. 21–23. Feulgen reaction of the spores of *B. cereus* after a few hours in Schiff's reagent. Photographed mounted in water, without counterstaining.

Figs. 24, 25. Spores of *B. cereus* fixed with osmium tetroxide vapour, hydrolysed with N-HCl at 60° and stained with Giemsa solution. Compare with Pl. 1, figs. 4, 5, and with Pl. 2, figs. 8, 9, 10, 11*b* and 12*b*. Note indistinct cytoplasm and transparent nuclei. In several spores the nuclei appear broken up into granules.

Fig. 26. Spores of *B. cereus* differentiated by method 3, stained with crystal violet and photographed in red light from a Wratten F 29 filter. Note that nuclei and cytoplasms appear more transparent than in the photographs on Pl. 2.

Fig. 27. Spores of *B. cereus* differentiated with N-HNO_3 and then treated with trypsin. The nuclei have been more or less completely destroyed. The cytoplasm has contracted. Stained with crystal violet.

PLATE 5. *B. subtilis* and *B. megatherium*

The magnification of the photographs on this plate is indicated by the scale beside fig. 30.

Figs. 28, 29. Two groups of spores of *B. subtilis* from a preparation that had been treated for 10 min. at room temperature with N-HNO_3 and was then stained with Giemsa solution. Compare these pictures with electron micrographs of treated and untreated spores of the same culture on Pl. 6.

Fig. 30. Spores of *B. megatherium* stained with crystal violet. Note a few deeply stained (prematurely germinated?) cells.

Fig. 31. Spores of *B. megatherium* treated for 10 min. with N-HNO_3 at room temperature. Very large nuclei are protruding from the cell surface, indicating considerable rearrangement of the spore contents. Giemsa stain.

PLATE 6

The magnification of the photographs on this plate is indicated by the scale beneath fig. 33.

Electron-micrographs of spores of the same culture of *B. subtilis* which provided figs. 28 and 29. The photographs on this plate were made by Dr C. G. Hedén in the Cell Research Laboratory of the Karolinska Institute, Stockholm, from specimens prepared by the writer in London, Canada.

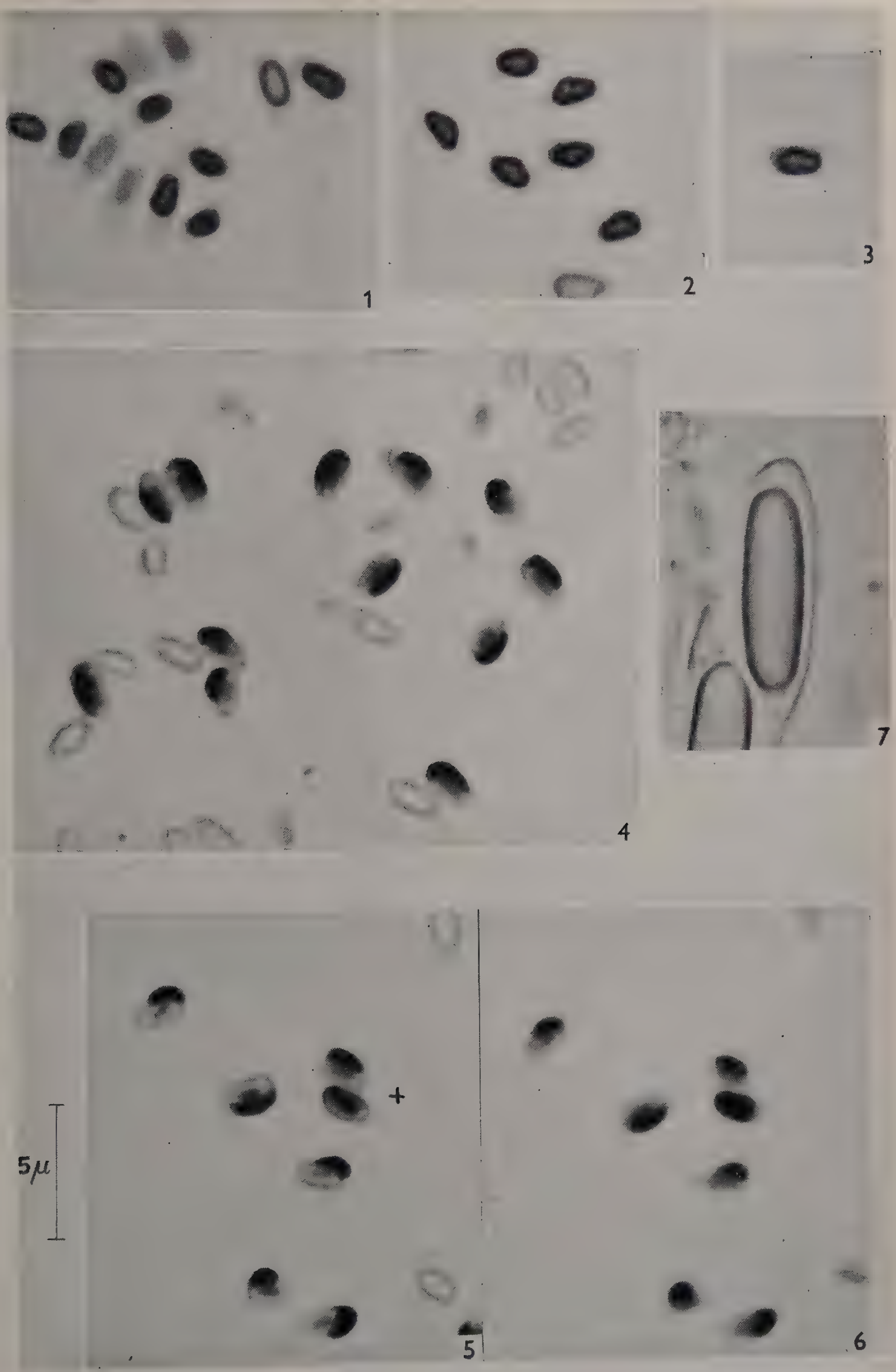


Fig. 1–7

C. F. Robinow—Structure of spores. Plate 1

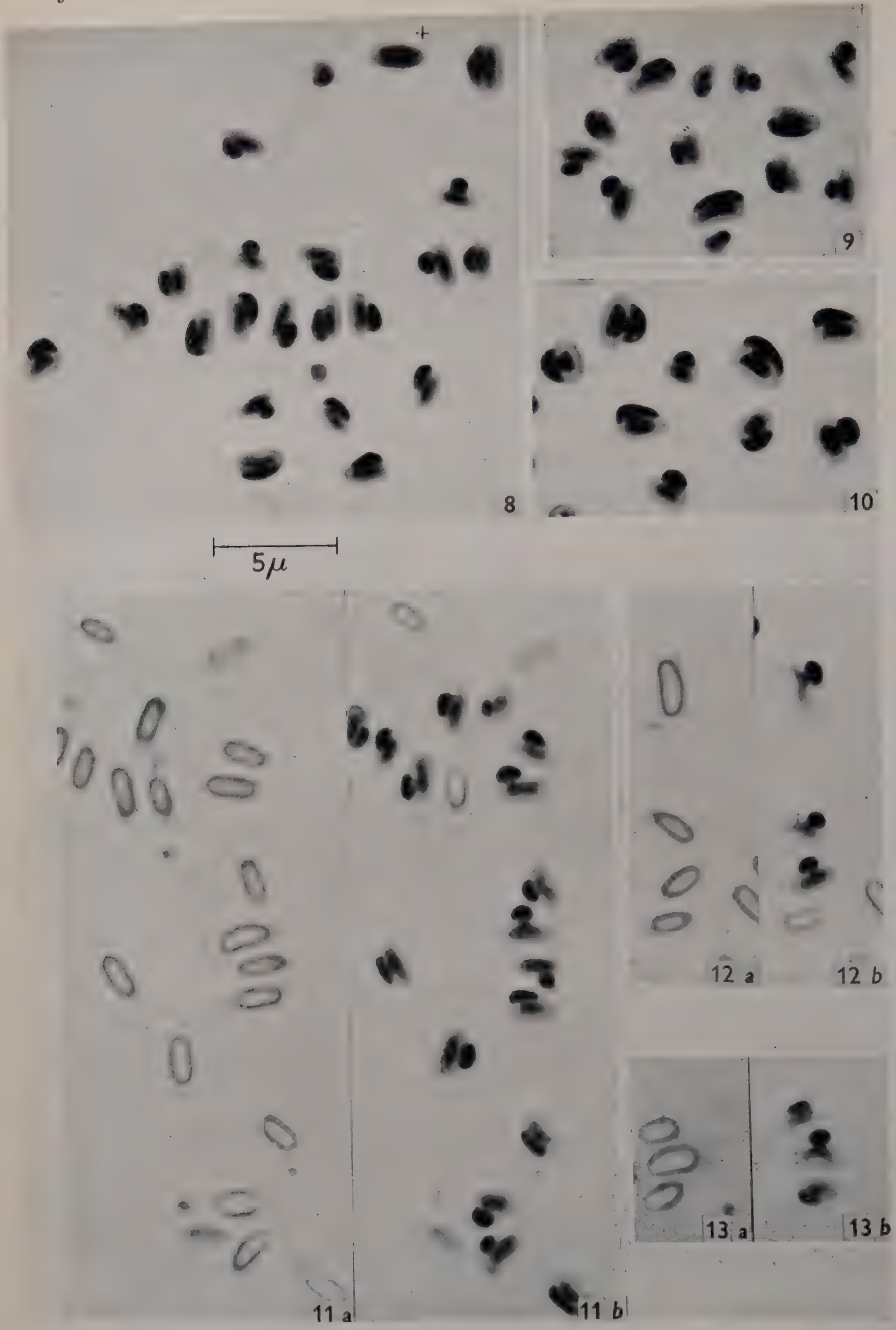
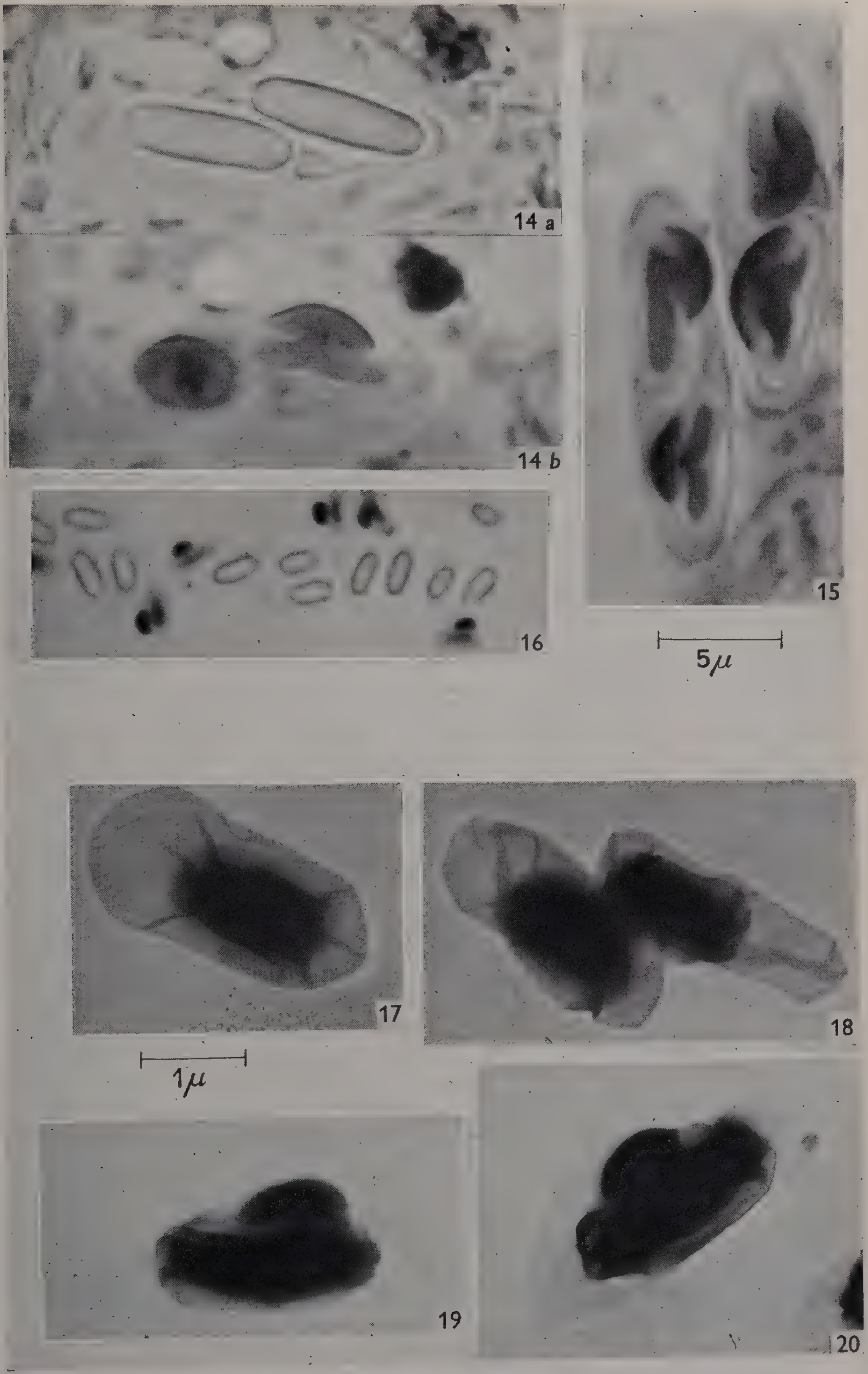


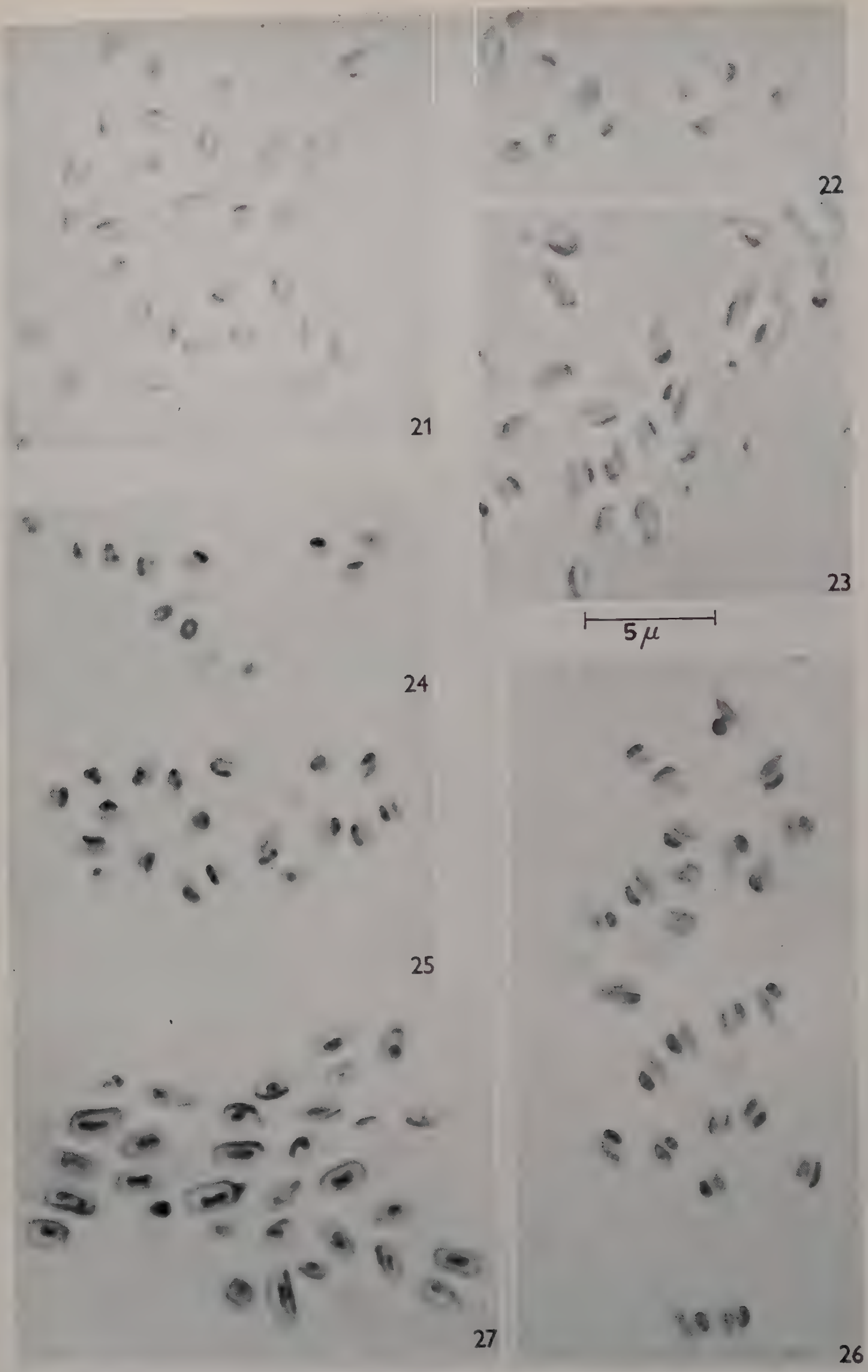
Fig. 8–13

C. F. Robinow—Structure of spores. Plate 2



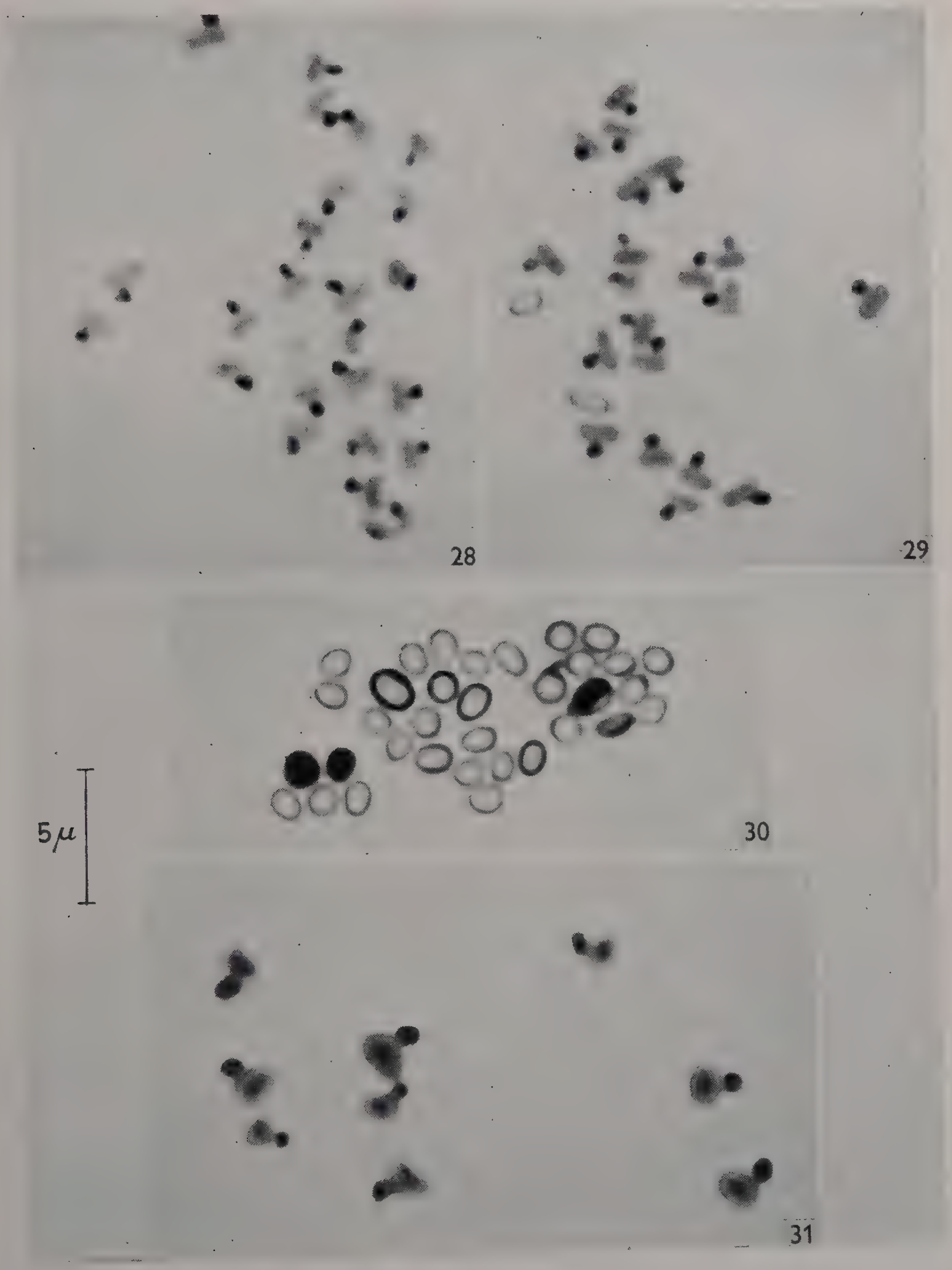
Figs. 14–20

C. F. Robinow—Structure of spores. Plate 3



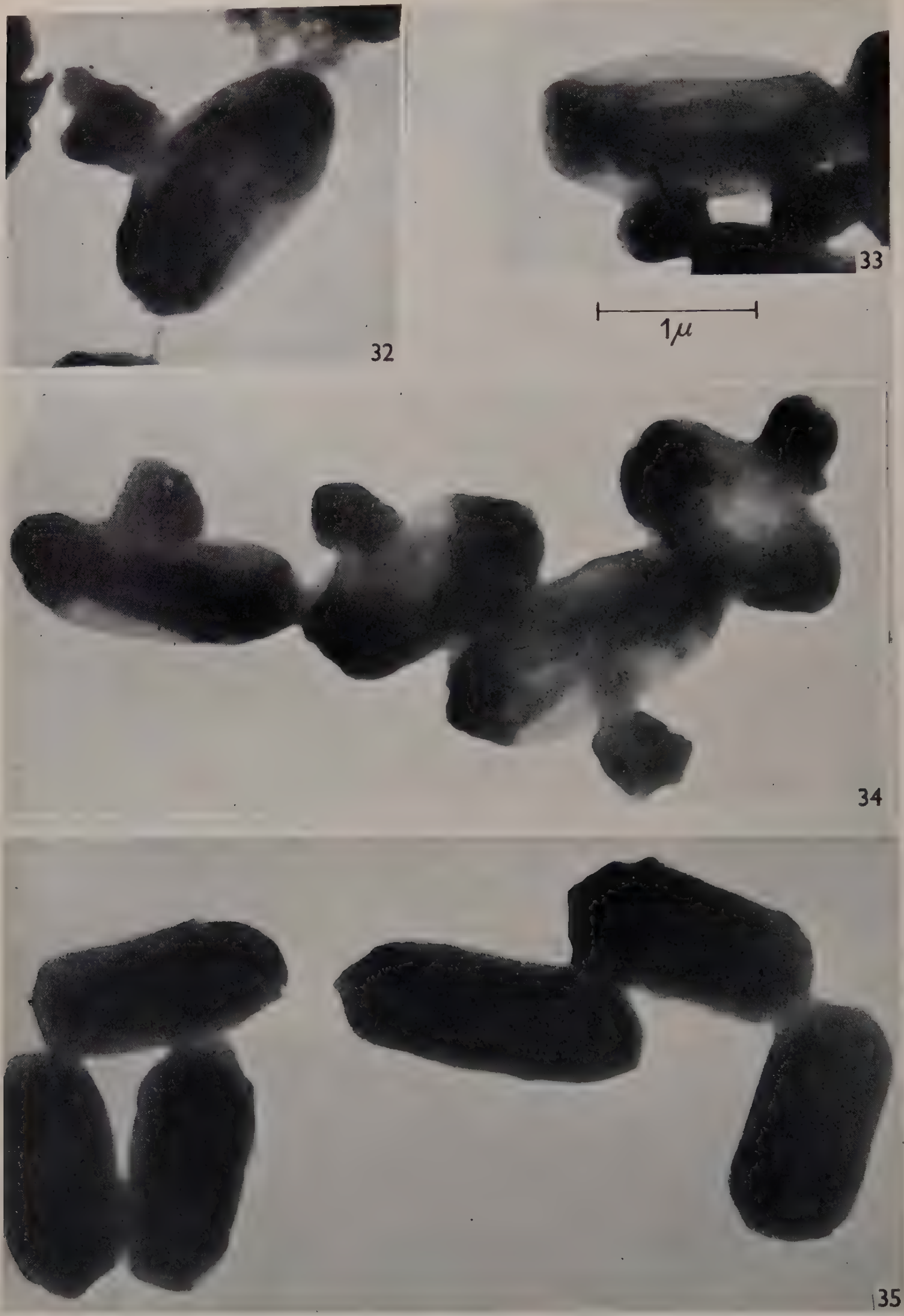
Figs. 21–27

C. F. Robinow—Structure of spores. Plate 4



Figs. 28–31

C. F. Robinow—Structure of spores. Plate 5



Figs. 32–35

C. F. Robinow—Structure of spores. Plate 6

Figs. 32–34. Spores from the same culture as those in fig. 28 treated for 20 min. with N-HNO_3 at room temperature, washed and dried. Note compact, angular nuclei, shrunken cytoplasm and delicate spore membrane. The ratio of nuclear volume to volume of the rest of the spore seems larger in light-optical pictures than in electron-micrographs. This is perhaps because the spores have to be dried for electron microscopy whereas they are mounted wet and are also imperfectly resolved in light-optical specimens.

Fig. 35. Untreated, resting spores of *B. subtilis*, washed and dried.

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Some Observations on Lysogenic Strains of Salmonella

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SUMMARY: The proportion of lysogenic cultures in five species of salmonella examined varied from 84 % in *Salmonella thompson* to 0 % in *S. pullorum.*

When estimating the amount of phage present in lysogenic cultures of *S. thompson* it was possible to obtain a plaque count approximately equal to the number of bacteria present in these cultures when the susceptible culture used for determining the counts was used relatively dilute. When used less dilute the counts were lower and equal to those obtained when the bacteria were killed by heating to 56° for 30 min. This lower figure is an estimate of the free phage in the culture.

Plaque size varied inversely as the amount of bacteria in the susceptible culture. When lysogenic cultures were used for counting a related phage, plaque size was diminished by partial interference.

Two types of relationship were noted between the growth rate of bacteria and phage in artificially induced lysogenic cultures of *S. thompson.* In one, the number of free phage particles was always directly related to the number of viable bacteria present; in the other, the phage particles increased rapidly in number during the logarithmic phase of bacterial multiplication and then decreased rapidly due presumably to their absorption by the bacteria.

The active infection of the lysogenic cultures with an unrelated phage usually resulted in an increased yield of the phage with which the culture was latently infected. In one instance the latent infection was transformed into an active one.

All the artificially induced resistant variant strains of *S. thompson* were lysogenic. It was impossible to make some cultures permanently resistant to two different phages as the resulting cultures were unstable and soon showed signs of active infection. Others were made with a difficulty not experienced during the making of singly resistant strains. Most of these were latently infected only with the first phage to which they had been made resistant; others carried both phages.

A considerable amount of work has been devoted to the study of active bacteriophage infection. Much less time has been spent on the examination of the host-parasite relationship in lysogenic bacteria, and in this field most of the work has been carried out on naturally occurring lysogenic strains. It has been shown that some of these strains are latently infected with more than one phage and that some bacteria may be made resistant to a phage, and yet not become latently infected with it. It is therefore obvious that critical studies should best be made on strains made lysogenic by laboratory procedures. For the preparation of these cultures, strains proved, as far as possible, to be non-lysogenic and not to have become resistant to phages after infection should be used; and from these strains variants prepared that are resistant to pure phages. The main purpose of this paper is to present some observations on such lysogenic cultures of salmonella.

MATERIALS AND METHODS

The identification of lysogenic strains. Lysogenic strains were identified by a modification of the cross-culture method (Fisk, 1942). Strains were grown in nutrient broth for 18 hr. at 37°; and each was used in turn as a 'basal' strain by spreading four drops (0·08 ml.) of the culture over the whole surface of a nutrient agar plate. The plates were then dried and the remaining cultures, after heating to 56° for 30 min., were 'spotted' on to it. This process of killing the bacteria in the cultures without destroying the phage that may be present is referred to below as heat treatment. The plates were incubated for 6–8 hr. at 37°, and left at room temperature overnight. Phage action was revealed the next morning by zones of complete or semi-complete lysis, or by discrete plaques. Modifications of this method were used when necessary or convenient. Sometimes one known susceptible strain only was used as the basal strain. When repeated tests were necessary to prove lysogenicity, young and 18 hr. cultures, both heat treated and alive, were used for spotting. Sometimes a number of strains were incubated together in broth and, after heat treatment, the product tested for phage.

Purification and propagation of phages. Since pure phage preparations can be obtained only by propagation on non-lysogenic strains (see below), phages were propagated on strains shown by repeated testing to be non-lysogenic. (The word 'strain' is used throughout with reference to bacteria and not to phages.) A suitably diluted suspension of a heat-treated lysogenic strain was mixed with a broth culture of the propagating strain and spread on nutrient agar and incubated at 37°. A discrete plaque was picked as soon as plaques were distinctly visible and replated with the propagating strain. This process was repeated twice. Finally, a discrete plaque with some of the surrounding bacterial growth was picked into broth, and incubated at 37° until lysis occurred. This was then heat treated, and tested for bacteriological sterility. The phages were designated by the number of the lysogenic strain from which the phage was derived followed by the number of the propagating strain.

Titration of phage preparations. Ten-fold dilutions of the phage preparations were made with quarter-strength Ringer's solution. Four drops (0·08 ml.) of an 18 hr. broth culture of a strain were spread over the surface of an agar plate, allowed to dry, and then spotted with a standard loopful of each dilution of the phage preparation. The plates were incubated at 37° for 18 hr., and read.

Preparation of phage-resistant strains. One drop of an 18 hr. broth culture of a susceptible strain was spread over a small area of the surface of an agar plate. When this was dry, one drop of an undiluted phage preparation was spotted on to the surface of the culture which was then incubated at 37°. A zone of complete lysis was observed after 4–5 hr. Incubation was continued until secondary growth appeared within this zone. A little of this secondary growth was picked off into broth, and incubated overnight, the culture plated on agar and a single colony picked and replated, finally a single colony from this plating was picked into broth, and incubated. The final culture was tested on agar to confirm its complete resistance to the undiluted phage preparation. Its colonial morphology was also examined. Only cultures that yielded colonies normal in appearance were retained; any forming colonies with a nibbled appearance were regarded as actively phage-infected and were discarded. Resistant strains were designated by the number of the susceptible strain followed by the number in parenthesis of the phage to which it had been made resistant. The words 'resistant strain' are used throughout this paper to mean phage-resistant strains.

Cross-resistance tests. These were carried out by a modification of Bail's (1923) original method. Resistant strains were prepared by the action of several phages on one bacterial strain. The phages were then titrated against each of the resistant strains in the manner previously described.

Table 1. *The occurrence of lysogenic strains in different species of salmonella*

Species	No. of strains tested	No. of lysogenic strains	Percentage of lysogenic strains
S. thompson	50	42	84
S. typhi-murium	50	13	26
S. dublin	67	14	20·9
S. enteritidis	20	4	20
S. pullorum	62	0	0

Bacterial counts. These were carried out by the method of Miles & Misra (1938).

Phage counts. A modification of the Miles & Misra (1938) method for bacterial counts was used for this purpose. Two per cent nutrient agar plates were dried for 24 hr. at 37° followed by a further 2 hr. with the lids raised. Four drops (0·08 ml.) of an 18 hr. broth culture of a susceptible strain were spread evenly over the surface of these plates, and drying was continued for another 1–1½ hr. with the lids raised. The area covered by the culture is referred to as the bacterial lawn. The fluid to be examined for its phage content was heat treated. The plates were incubated at 37° for 18 hr. before plaque counts were made.

All the bacterial cultures used were smooth unless otherwise stated. The expressions 'lysogenic', carrying phage', and 'latently infected with phage' are used synonymously.

RESULTS

The occurrence of lysogenic strains amongst different species of salmonella

Representative species of salmonella were examined for lysogenicity. The tests were carried out within species only, e.g. the *Salmonella enteritidis* strains were only tested for lysogenicity to other strains of that species. Table 1 illustrates the considerable difference in the proportion of lysogenic strains within different species, varying from 84 % for *S. thompson* to 0 % for *S. pullorum*. The figures for *S. enteritidis* (20 %) are considerably lower than those, for example, of Burnet (1932) who found fifteen of eighteen smooth strains to be lysogenic. Because Burnet found a strain of *S. gallinarum* to be a very suitable indicator for these lysogenic strains, six strains of *S. gallinarum* were used as indicators for the twenty *S. enteritidis* strains tested. They did not reveal any more strains to be lysogenic. It is probable, however, that if a more thorough search for suitable indicator strains were made, more strains would be shown to be lysogenic. Since a high proportion of the *S. thompson* cultures were lysogenic, and as a preliminary examination of the phages carried by some of these cultures showed many of the phages to be different from each other, it was decided to use the *S. thompson* strains for more detailed studies.

Nine phages isolated from lysogenic cultures of *S. thompson* were titrated against the fifty strains of *S. thompson* referred to in Table 1. Of the eight non-lysogenic strains, six were resistant in varying degrees to the phages, and two, strains 19 and 30, were fully susceptible to all the nine phages. Thus strains 19 and 30 appeared to be non-lysogenic, and to have acquired no resistance to bacteriophages and were therefore ideal for this investigation.

Most of the nine phages had been propagated on lysogenic strains. Consequently, they were contaminated with phages contributed by the strains; and were accordingly purified and propagated on strain 19.

Variants resistant to these nine phages were prepared from strain 19; all were lysogenic. By cross-resistance tests six of these phages, 1/19, 2/19, 5/19, 7/19, 8/19 and 13/19, were shown to be quite different from each other. One interesting feature of some of these tests was that of partial interference, as shown by the appearance of plaques of subnormal size in some of the titrations. These plaques were often quite minute and difficult to discern.

The growth rate of bacteria and phage in lysogenic strains

It was necessary to estimate the probable phage mortality during heat treatment. Four of the phages were freed from bacteria by Seitz filtration. One portion of the filtrate was heated to 56° for 30 min. Phage counts of both portions (Table 2) indicated that the loss during heating was negligible.

Table 2. *The effect of heating phage preparations to 56° for 30 min.*

	Phage particles, 10^4/ml.	
Phage	Before heating	After heating
7/19	70	80
8/19	48	38
2/19	28	20
5/19	28,000	30,000

Nutrient broth at 37° in 20 ml. amounts was seeded with 0·1 ml. of an 18 hr. culture of a lysogenic strain and incubated at 37°: 0·1 ml. samples were removed at intervals and used for bacterial and phage counts, the phage counts being made of an unheated and a heat-treated portion, upon plates seeded with strain 19. Seven lysogenic variants of strain 19 carrying different phages were examined. The variants, apart from differences in the yield of phage, could be classified into two types, types 1 and 2, containing respectively three and four variants.

Type 1. The results obtained with strain 19 (5/19) are representative of type 1 (Table 3 and Fig. 1). The phage population of the culture was directly related to the number of visible bacteria present, both in the logarithmic phase

Table 3. *The growth rate of bacteria and phage in culture* 19 (5/19)

Time	Viable bacteria, 10^6/ml.	Viable phage particles, 10^6/ml. Using live culture	Viable phage particles, 10^6/ml. Using heat-treated culture
0 hr.	4·2	0·21	0·2
½ ,,	5·2	0·23	0·25
1 ,,	5·5	0·31	0·3
1½ ,,	12·0	1·0	0·6
2 ,,	28·0	1·2	0·85
2½ ,,	50·0	—	1·7
3 ,,	80·0	7·3	5·5
3½ ,,	230·0	19·0	12·0
4 ,,	340·0	29·0	22·0
4½ ,,	450·0	37·0	32·0
5 ,,	—	—	46·0
6 ,,	550·0	50·0	52·0
8 ,,	400·0	70·0	90·0
10 ,,	520·0	85·0	90·0
12 ,,	500·0	65·0	65·0
14 ,,	490·0	56·0	100·0
16 ,,	480·0	50·0	78·0
18 ,,	750·0	70·0	85·0
24 ,,	1400·0	110·0	100·0
4 days	500·0	100·0	250·0
7 ,,	120·0	130·0	150·0
15 ,,	90·0	35·0	40·0
25 ,,	40·0	42·0	36·0

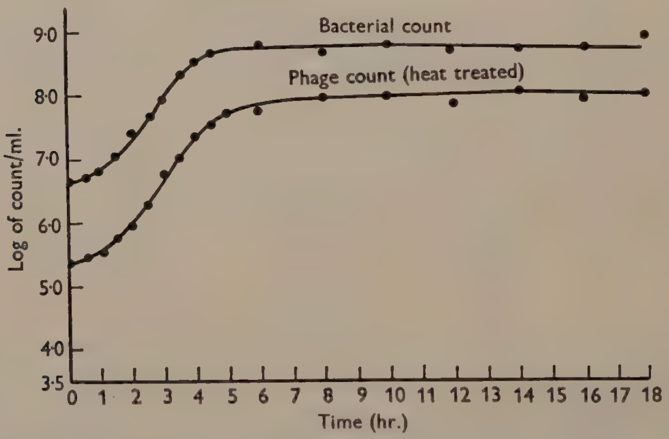

Fig. 1. The growth rate of bacteria and phage in the lysogenic culture 19 (5/19).

and in the stationary bacterial phase. As the number of viable bacteria decreased, the number of phage particles also decreased but at a slower rate. This experiment was repeated with similar results. Another three variants of strain 19 resistant to phage 5/19 and two variants of the other non-lysogenic strain 30 resistant to this phage behaved in several tests like the original strain 19 (5/19) although the yield of phage varied.

Type 2. The results obtained with strain 19 (1/19) are representative of type 2 (Table 4 & Fig. 2). In this case the initial inoculum contained a small amount of phage, but, as the culture entered the logarithmic phase, the phage increased more rapidly than the bacteria. At the end of the bacterial logarithmic phase, the number of phage particles rapidly decreased by about 90 % in 1 hr. and then decreased much more slowly. This experiment was repeated twice with similar results. The occurrence of a peak in the phage count followed by a rapid decline was found to be independent of the time of incubation, but dependent on the number of multiplying bacteria present, the peak occurring near the end of the logarithmic phase when the culture contained approximately 30×10^7 bacteria/ml.

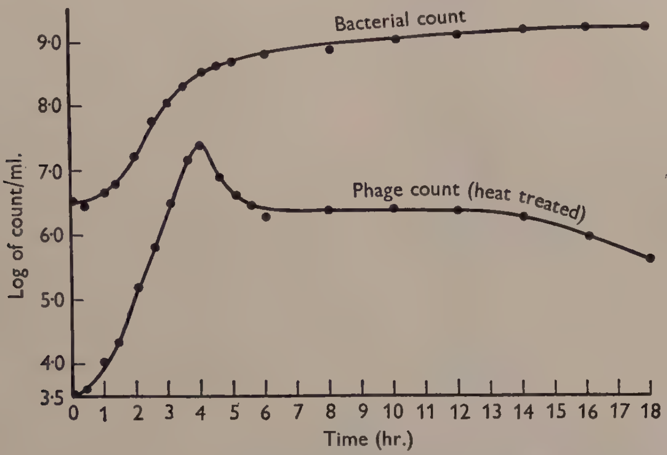

Fig. 2. The growth rate of bacteria and phage in the lysogenic culture 19 (1/19).

Another two variants of strain 19 resistant to phage 1/19 and two variants of strain 30 resistant to this phage were also like the original strain 19 (1/19).

Three variants resistant to phage 1/19, strains 2 (1/19), were also prepared from a naturally occurring lysogenic strain, strain 2. Bacterial and phage counts were made on cultures after 3 and 18 hr. incubation (Table 5). Phage counts were made using strain 2 as the bacterial lawn to estimate the amount of 1/19 present and using strain 19 (1/19) to estimate the amount of the strain 2 phage. At 18 hr., though not at 3 hr., variant 2 (1/19)*a* and 2 (1/19)*c* differed in phage production from 2 (1/19)*b* which contained 100 times more phage of each kind than the other two. It is noteworthy that the amount of phage present in strains 19 (1/19) and strain 2, which are each carrying only one type of phage, was greater than the total present in cultures of the variants carrying the two phages. When these experiments were repeated the results were similar.

Phage counts of lysogenic strains using live instead of heat-treated cultures

The similarity of phage counts of living and heat-treated lysogenic strains (Tables 3 and 4) is surprising, if it is assumed that all bacteria in a lysogenic culture contain phage, or phage precursor, which might be expected to be released from living but not dead cultures. The counts, however, represent only free phage. Phage counts on heat-treated and live cultures and on centrifuged live 18 hr. cultures of strain 19 (5/19) were also similar being respectively 85, 70 and 73 millions per ml. The centrifuged deposit of bacteria obtained after

Table 4. *The growth rate of bacteria and phage in culture* 19 (1/19)

Time	Viable bacteria, 10^6/ml.	Viable phage particles, 10^6/ml. Using live culture	Using heat-treated culture
0 hr.	3·0	0·004	0·0032
½ ,,	2·7	—	0·0044
1 ,,	4·4	—	0·015
1½ ,,	5·7	0·05	0·033
2 ,,	15·0	0·17	0·18
2½ ,,	55·0	0·48	0·8
3 ,,	120·0	2·6	3·8
3½ ,,	220·0	11·0	15·0
4 ,,	300·0	16·0	22·0
4½ ,,	380·0	—	7·6
5 ,,	450·0	2·0	3·1
5½ ,,	600·0	1·9	2·6
6 ,,	550·0	—	2·0
8 ,,	800·0	3·6	3·0
10 ,,	1000·0	—	3·0
12 ,,	1100·0	—	2·8
14 ,,	1900·0	—	1·7
16 ,,	—	—	0·8
18 ,,	1600·0	0·54	0·4
5 days	120·0	—	0·52

Table 5. *Phage counts of variants of the lysogenic strain* 2, *resistant to phage* 1/19

Identification of strain	Count of phage	Phage particles, 10^4/ml. of culture 3 hr.	18 hr.
2 (1/19)*a*	1/19	34·0	0·12
2 (1/19)*b*	1/19	45·0	30·0
2 (1/19)*c*	1/19	35·0	0·2
19 (1/19)	1/19	260·0	40·0
2 (1/19)*a*	Phage carried by strain 2	190·0	2·2
2 (1/19)*b*	Phage carried by strain 2	250·0	200·0
2 (1/19)*c*	Phage carried by strain 2	200·0	2·0
2	Phage carried by strain 2	2200·0	2400·0

centrifuging the live culture was washed in quarter-strength Ringer's solution several times and then made up to the original volume of culture with broth. The phage count was now only 70,000 particles per ml. although there was little alteration in the bacterial count. Thus, *under the condition of test*, the counts were uninfluenced by the phage carried within the bacteria cells and represent free phage only.

However, when counting phage on living cultures of 19 (1/19) on rather moist bacterial growth, two kinds of plaque appeared. One type was large and characteristic of phage 1/19, and in numbers similar to those in heat-treated cultures. The other was of small size. Well-dried plates were now seeded with strain 19, but the count dilutions were dropped on to the agar immediately the inoculum was dry. Both live and heat-treated culture, and the supernatant fluids of these cultures after centrifuging, were counted (Table 6). The count of the small plaques is probably underestimated, since some of them were very difficult to discern. It was obvious that the increase in phage count due to the plaques of subnormal size was related to the presence of live bacteria, and it seemed probable that the multiplication of these bacteria after being dropped on the lawn of susceptible culture resulted in the release of phage particles which were responsible for the increased count. The fact that these small plaques were not apparent on plates used after the bacterial lawn had been allowed to dry and incubate for 1–1½ hr. may be accounted for by assuming that phage liberated during multiplication of the lysogenic bacteria is unable to form plaques in the much denser bacterial growth present after 1–1½ hr. incubation of the lawn.

Table 6. *Plaque counts of an* 18 *hr. broth culture of strain* 19 (1/19) *using a bacterial lawn unincubated before carrying out the counts*

	No. of normal plaques, 10^4/ml.	No. of small plaques, 10^4/ml.
Live broth culture	85	400
Supernatant of centrifuged broth culture	100	0
Heat-treated broth culture	87	0
Supernatant of centrifuged heat-treated broth culture	75	0

The relation of plaque count and the time of incubation of the bacterial lawn before spotting on the count dilutions was now determined. Phage counts were made of cultures of 19 (5/19) and 19 (1/19), and supernatant fluids of these cultures after centrifuging upon plates of strain 19 incubated for different periods. These plates had been well dried before sowing the bacterial lawns and the count dilutions were quickly absorbed, so that extra plaques formed would have developed from phage particles liberated by developing colonies of the lysogenic bacteria, one plaque representing one colony (Table 7). Live broth cultures of strains 19 (5/19) and (1/19) gave higher plaque counts when seeded immediately after sowing the bacterial lawn or after ½ hr. incubation, than those of free phage present in the supernatant fluids of the centrifuged cultures, and higher than those obtained on agar plates that had been incubated for 1 hr. or more. The higher count can be accounted for by plaques of subnormal size. Counts on lawns incubated for 1–1½ hr. correspond to the free phage present (cf. Tables 3 and 4). With 2–3 hr. incubation before 'spotting' the plaques size was considerably smaller, often pin-point in size, making counting difficult, and showing that the amount of bacterial lawn has a pronounced effect on plaque size. With 4 hr. incubation the amount of bacterial lawn was such that no plaques became visible.

Since the amount of bacterial lawn influenced the phage counts, an attempt

Table 7. *The effect of incubating the bacterial lawn on phage count plates before seeding with live lysogenic bacteria*

Time of incubation of bacterial lawn (hr.)	3 hr. culture 19 (5/19)				3 hr. culture 19 (1/19)			
	Untreated		Supernatant fluid after centrifugation		Untreated		Supernatant fluid after centrifugation	
	Plaques, 10^5/ml.	Type of plaque	Plaques, 10^5/ml.	Type of plaque	Plaques, 10^5/ml.	Type of plaque	Plaques, 10^5/ml.	Type of plaque
0	270	L, S	150	L	1200	L, S	25	L
$\frac{1}{2}$	250	L, S	130	L	650	L, S	27	L
1	170	L	160	L	9	L	25	L
$1\frac{1}{2}$	180	L	150	L	23	L	20	L
2	150	S	200	S	20	L	30	L
$2\frac{1}{2}$	100	S	60	S	15	S	20	S
3	60	S	0	—	25	S	23	S
4	0	—	0	—	0	—	0	—

L = plaques of normal size. S = plaques of subnormal size.

was made to use lawns on which phage counts of a live lysogenic culture were equal to the number of bacteria. Accordingly, bacterial lawns were sown from different dilutions of an 18 hr. broth culture of strain 19, and used immediately the lawns were absorbed for phage counts of 3 and 18 hr. live broth cultures of strains 19 (1/19) and 19 (5/19) (Table 8). With the 1/10 dilution the phage counts almost equal the viable count of the bacteria, showing that each bacterium was capable of exhibiting its lysogenic state under the conditions of test. The plaque counts of similar but heat-treated cultures were independent of the amounts of bacterial lawn, though the plaques were larger on the thinner lawns. On the 1/20 lawns accurate plaque counts were difficult.

Table 8. *The effect of using different dilutions of a broth culture of strain* 19 *as the bacterial lawn on phage counts of live lysogenic cultures*

Culture counted	Bacteria, 10^6/ml.	Plaques, 10^6/ml. on lawns seeded from culture diluted			
		1/1	1/5	1/10	1/20
3 hr. broth culture of 19 (5/19)	150·0	55·0	45·0	100·0	90·0
18 hr. broth culture of 19 (5/19)	1000·0	170·0	450·0	300·0	350·0
3 hr. broth culture of 19 (1/19)	71·0	20·0	42·0	55·0	—
18 hr. broth culture of 19 (1/19)	1200·0	120·0	350·0	1000·0	930·0
Heat treated 18 hr. culture of 19 (1/19)	0	0·9	0·75	0·8	0·45

The bacterial lawns were not incubated before use.

As a result of these experiments, subsequent phage counts were made on heat-treated bacterial cultures.

The active infection of lysogenic cultures with other phages

Burnet & McKie (1929) infected a lysogenic culture of *S. enteritidis* with another phage and found that this active infection decreased the yield of phage carried by the lysogenic culture. They concluded that lysis by an unrelated phage diminishes the amount of latent phage liberated, and that there is no evidence that the latent phage exists within the lysogenic bacteria as an independent unit.

The experiments were repeated with the lysogenic variants of strain 19. The technique consisted of adding 0·1 ml. of an 18 hr. broth culture of a lysogenic strain to each of two tubes containing 20 ml. of broth. The tubes were incubated at 37° and, after a suitable previously determined period, an amount of phage, to which the strain was susceptible, was added to one tube and a similar volume of broth to the other. The number of phage particles added was usually about 5–10 times the number of bacteria estimated to be present at the time of addition of the phage preparation. Bacterial and phage counts were made of both cultures immediately before the addition of the infecting phage to confirm that they contained approximately equal numbers of bacteria which were liberating approximately equal numbers of latent phage particles. Incubation was continued and phage counts were made at intervals on 0·1 ml. samples after heat treatment of both infecting and latent phage using lawns of the appropriate susceptible strains. The procedure is best illustrated by an example. Strain 19 (2/19) was actively infected with phage 5/19. The yield of the infecting phage, 5/19, was measured on strain 19 (2/19) and the latent phage, 2/19, on strain 19 (5/19). The counts were also carried out using strain 19 as lawn. The two phages, 2/19 and 5/19, were previously titrated on strains 19 (2/19), 19 (5/19) and 19 to confirm that 2/19, for example, was without effect on strain 19 (2/19) and to determine whether the titration on 19 (5/19) revealed any degree of interference when compared with the same titration on 19. The amounts of the two phages after lysis of the lysogenic cultures by the infecting phages are shown in Table 9. The results with phage 13/19 and strain 19 (7/19) are shown in more detail in Table 10 and in Fig. 3, as they are typical of most of the experiments; and those with phage 5/19 on strain 19 (1/19) in Table 11 and Fig. 4, on account of their exceptional interest.

Table 9. *The active infection of lysogenic cultures with other phages*

Lysogenic culture	Phages used for active infection	Infected culture (particles, 10^5/ml.) Infecting phage	Latent phage	Control culture (particles, 10^5/ml.), latent phage
19 (2/19)	5/19	7,000·0	780·0	55·0
19 (2/19)	1/19	8,200·0	850·0	28·0
19 (1/19)	2/19	20,000·0	30·0	3·5
19 (7/19)	13/19	15,000·0	410·0	23·0
19 (13/19)	7/19	20,000·0	100·0	110·0
19 (5/19)	1/19	12,000·0	150·0	130·0
19 (1/19)	5/19	250·0	12,000·0	150·0

In the first four experiments in Table 9, active infection with an unrelated phage greatly increased the amount of latent phage liberated from the lysogenic culture by between nine- and thirty-fold in different cases. There was no increase in Exps. 5 and 6, but it is possible that the increase in these cases is masked by the early destruction of some of the lysogenic bacteria by the active phage infection. Exp. 7 is particularly interesting, as the addition of phage 5/19 appears to have stimulated an active infection in strain 19 (1/19) with phage, 1/19, with which it was previously only latently infected; large amounts

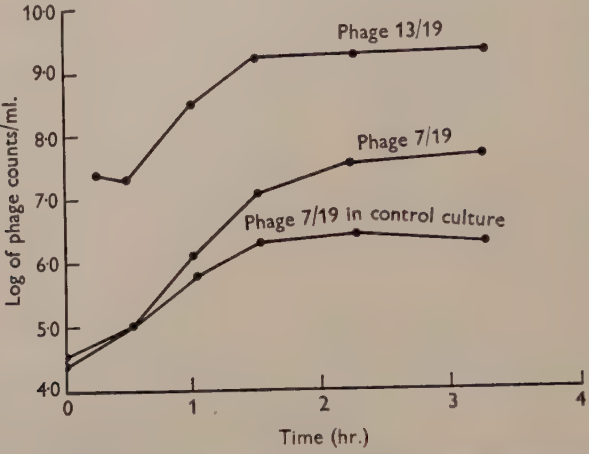

Fig. 3. The active infection of the lysogenic culture 19 (7/19) with phage 13/19. Phage 13/19 was added at 0 hr. The control culture of 19 (7/19) was not inoculated with phage 13/19.

Table 10. *The effect of infection by phage* 13/19 *on strain* 19 (7/19)

Time	Infected culture (particles, 10^5/ml.) Infecting phage	Infected culture (particles, 10^5/ml.) Latent phage	Control culture (particles, 10^5/ml.), latent phage
Before addition of phage	0	0·23	0·2
¼ hr. after addition	230·0	—	—
½ ,, ,,	180·0	1·0	1·0
1 ,, ,,	3,000·0	15·0	7·5
1½ ,, ,,	17,000·0	120·0	20·0
2¼ ,, ,,	19,000·0	350·0	25·0
3¼ ,, ,,	15,000·0	410·0	23·0
18 ,, ,,	30,000·0	2,800·0	140·0

Table 11. *The effect of infection by phage* 5/19 *on strain* 19 (1/19)

Time	Infected culture (particles, 10^5/ml.) Infecting phage	Infected culture (particles, 10^5/ml.) Latent phage	Control culture (particles, 10^5/ml.), latent phage
Before addition of phage	0	4·4	3·5
¼ hr. after addition	120·0	3·0	2·5
½ ,, ,,	80·0	4·5	8·0
¾ ,, ,,	120·0	7·0	23·0
1 ,, ,,	220·0	3,000·0	38·0
1½ ,, ,,	250·0	12,000·0	150·0
2¼ ,, ,,	270·0	11,000·0	120·0
18 ,, ,,	100·0	12,000·0	2·0

of the phage 1/19, 12×10^8 particles/ml., were present after lysis, but the amount of the infecting phage, 5/19, remained relatively unchanged at 25×10^6 particles/ml. (see also Table 11 and Fig. 4). Phage 5/19 and phage 1/19 produced quite different types of plaques when grown on strain 19; the plaques of 1/19 were clear with a sharp edge and those of 5/19 were semi-clear with a considerable amount of resistant growth and ill-defined edge. Thus it was possible to confirm the counts obtained using 19 (1/19) and 19 (5/19) as lawns by counts of the two plaque types on strain 19 instead. The experiment was also repeated with similar results. It is noteworthy that although phage 5/19 was able to induce an active infection of strain 19 (1/19) by its latent phage, it was unable to do so in strain 19 (2/19); and phage 2/19 was similarly unable to stimulate an active infection of strain 19 (1/19) with its latent phage 1/19. The five phages used in the above experiment were used to infect the non-lysogenic strain 19 under exactly the same conditions; the yield of phage was similar to that from the lysogenic strains with the exception, of course, of the yield of 5/19 on 19 (1/19).

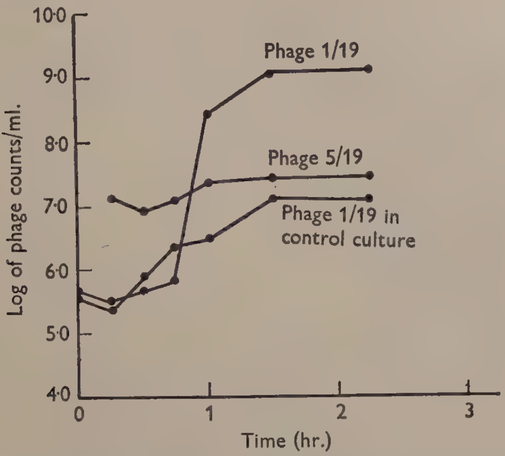

Fig. 4. The active infection of the lysogenic culture 19 (1/19) with phage 5/19. Phage 5/19 was added at 0 hr. The control culture of 19 (1/19) was not inoculated with phage 5/19.

Observations on bacterial cultures rendered resistant to more than one phage

The resistant variants of strain 19 were made resistant to other phages. The preparation of doubly-resistant strains was, unlike that of singly-resistant strains, not without considerable difficulty. Lysis by the second phage occurred in the usual way but, when the resistant growth produced by continued incubation was plated, the resulting colonies were often of the nibbled variety, indicating that they were actively infected with phage. Broth cultures from these colonies were proved to contain both phages, and when they were replated, yielded normal as well as nibbled colonies; cultures from these normal colonies were now fully susceptible to the second phage and were shown to carry only the first phage to which they had been made resistant. The true doubly-resistant variants were plated several times before determining the phages with which they were now latently infected. The determination of latent infection is best illustrated by an example. Strain 19 (1/19) was made resistant to phage 2/19. Cultures of the doubly-resistant variant that resulted were examined at different periods of incubation; plate lysis of strain 19 (1/19) by these cultures after heat treatment was taken to indicate the presence of phage 2/19, and lysis of strain 19 (2/19) as indicative of the presence of phage 1/19. The doubly-resistant strains were numbered thus: strain 19 (1/19 + 2/19), the phage to which strain 19 was first made resistant, preceding the phage, 2/19, to which it was subsequently made resistant. The results of some of these experiments are given in Table 12. With the exception of strain 19 (2/19 + 5/19) the doubly-resistant variants carried only the first phage to which they had been made resistant. The six types of variants were subcultured several times over a period of 3 months, single colonies being picked on each occasion; no change of resistance or phage-carriage was noted. It was originally intended to make eight doubly-resistant strains, but it was found impossible despite repeated attempts to make permanent resistant variants of strain 19 (7/19) to phage 13/19 and strain 19 (1/19) to phage 5/19. The only kind of resistant culture produced in these cases was of the nibbled colony type which after a few subcultures broke down to yield colonies which did not carry the second phage and which were fully susceptible to it.

Table 12. *The carriage of phages by strains made resistant to two phages*

Doubly-resistant variant*	No. of variants tested	Phages carried
19 (1/19+2/19)	21	1/19
19 (2/19+1/19)	18	2/19
19 (13/19+7/19)	13	13/19
19 (2/19+5/19)	1	2/19, 5/19
19 (5/19+2/19)	1	5/19
19 (5/19+1/19)	15	5/19

* The phages to which the strains were first made resistant are placed first in the brackets.

Naturally occurring lysogenic cultures were made resistant to other phages to determine whether their status was similar to that of the artificial doubly-resistant strains. They were equally difficult to make. Of five doubly-resistant variants, two were resistant to, but did not carry, the phage to which they had been made resistant.

DISCUSSION

There is much conflicting evidence about the absorption of homologous phage by phage-resistant strains; some workers contend that these strains do absorb phage while others deny it. My results suggest that both contentions are correct: some strains, e.g. strain 19 (1/19), absorbed the phage whereas other strains appeared to be unable to do so. The occurrence of absorption appeared to depend more on the phage than on the bacterium; all the lysogenic variants of strain 19 resistant to the same phage or to phages shown to be identical by cross-resistance tests exhibited the same kind of phage-bacterium growth curve, and variants of other strains (e.g. strains 30 and 2) resistant to the same phages behaved in a similar fashion.

A most interesting effect was obtained when a lysogenic culture was used as the bacterial lawn. When the carried phage was identical with, or closely similar to, the phage that was being counted, there were, of course, no plaques; and again, when the phage carried by the lysogenic culture was unrelated to the phage that was being counted, the plaques were similar in size to those obtained when a non-lysogenic culture was used as the lawn. However, when the lysogenic culture carried a phage related to the phage being counted, partial interference often resulted, indicated by the appearance of plaques of subnormal size. A variation of the conditions of test, such as an increase in the amount of bacterial lawn, was sometimes sufficient to inhibit the formation of such plaques. As some of the methods of phage typing depend on the pattern of reactions that some strains show when tested with a number of phages, it is apparent that the relationship of the phages carried by lysogenic strains being typed to the typing phages can account for some strains being regarded as separate types and can also be confusing unless a strict standard method of test be followed.

The observations on the active infection of lysogenic strains do not agree with the conclusion of Burnet & McKie (1929) that lysis by an unrelated phage diminishes the amount of latent phage liberated by a lysogenic strain; usually there was a considerable increase in the yield of phage. The number of latent phage particles liberated by some of the lysogenic strains was probably approximately equal to the number of bacteria present in the lysogenic culture before lysis by the unrelated phage. This, however, could not be confirmed owing to the impossibility of counting the number of *viable* bacteria that had been actively infected with phage. In fact, the results support the view that phage may exist within the bacterial cell as an intrinsic unit rather than as, or in addition to, some form of phage precursor. The stimulation of an active infection in the lysogenic culture 19 (1/19) with the phage it was carrying, 1/19, by the addition of phage 5/19 was particularly interesting, as it bears some resemblance to the change from latent to active infection that may sometimes occur in virus diseases of animals.

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The Typing of *Salmonella thompson* by Means of Bacteriophage

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SUMMARY: By the use of seven phage preparations, forty-one of fifty smooth strains of *Salmonella thompson* were classified into eleven types. The remaining nine strains were insusceptible to all the test phages; mouse passage did not render them susceptible. Acquired phage resistance was responsible for many of the strains being regarded as different phage types. A study of phage-resistant variants cast doubt on the complete validity of Bail's cross-resistance tests. The danger of using lysogenic cultures for the propagation of phages for the study of phage mutation were noted. Most of the *S. thompson* phages were fully active against strains of *S. cholerae-suis* but not for other *Salmonella* sp. that were tested.

In a recent study (Williams Smith, 1951) a number of bacteriophages were isolated from lysogenic strains of *S. thompson*. The purpose of this present paper is to report observations that were made while evolving a method of typing strains of *S. thompson* by the use of these phages.

MATERIALS AND METHODS

The method of isolation, purification, and propagation of the phages used in this study, and the method of titration of the final phage preparations, the preparation of phage-resistant strains and the differentiation of the phages from each other by cross-resistance tests have been reported elsewhere (Williams Smith, 1951).

Method of setting-up tests. A modification of the method devised by Wilson & Atkinson (1945) for the phage typing of staphylococci was used. Nutrient agar plates containing 2 % agar were dried at 37° for 2 hr. with the lids partly raised. Four drops (0·08 ml.) of an 18 hr. broth culture of the strain to be typed were spread evenly over the surface of a plate by means of a glass spreader. After the plates had dried, a standard loopful of each phage preparation in test dilution, i.e. the highest dilution capable of producing confluent lysis of its propagating strain, was spotted on to the plate. When several strains were tested, the phage preparations were spotted on to each plate by means of a dropping pipette (150 drops/ml.). For the sake of convenience, the bottom of each plate was marked into a number of squares equal to the number of phage preparations in use, by means of a grease pencil. When the drops had been absorbed, the plates were incubated at 37° overnight, and read next morning.

All the phage preparations were purified and propagated on strain 19, which, as far as could be determined, was non-lysogenic and did not show any evidence of acquired phage resistance. Each phage was designated by the number of the lysogenic strain from which it was isolated, followed by the number of the strain upon which it was propagated, i.e. strain 19. One of the phage preparations, 6/19, was used in two dilutions, one 100 times stronger than the normal test dilution, as it was found that the stronger preparation was capable of lysing some strains which would otherwise be considered untypable. The strong preparation for convenience was designated 6/12/19 simply because in this strength it has been found capable of lysing strain 12, a strain previously regarded as untypable.

RESULTS

The results of the phage typing of fifty smooth strains of *S. thompson* by means of seven phage preparations are shown in Table 1. Of the fifty strains, forty-one (82 %) were typable and nine (18 %) were not acted upon by any of the phages. The forty-one typable strains could be divided into eleven types. Apart from one type, type 9, which comprised 28 % of the whole, the number of other types were not large. The fifty strains were retested several times during a period of 6 months and, apart from the variations inherent in any method of phage-typing which depends on the pattern reactions given by strains with a number of test phages, they showed no significant variation in their susceptibility. During this period they were subcultured a number of times. Thus, judging only from strains maintained under laboratory conditions, the different phage types appeared to be relatively stable.

Table 1. *The phage typing of fifty strains of* Salmonella thompson

Phage type	Phage preparations							Strains of *S. thompson*	
	7/19	5/19	8/19	13/19	2/19	6/19	6/12/9	No.	%
1	CL	CL	CL	CL	CL	CL	CL	2	4
2	–	CL	CL	CL	CL	CL	CL	1	2
3	–	–	CL	CL	CL	CL	CL	8	16
4	–	CL	–	CL	CL	CL	CL	1	2
5	–	–	–	CL	CL	CL	CL	1	2
6	–	CL	+	–	CL	CL	CL	3	6
7	CL	–	CL	–	CL	+	CL	2	4
8	CL	–	–	+	–	CL	CL	1	2
9	–	–	–	–	–	CL	CL	14	28
10	–	–	–	–	–	–	CL	6	12
11	CL	–	+	–	+	–	++	2	4
Untypable	–	–	–	–	–	–	–	9	18

Confluent lysis = CL; semi-confluent plaques = ++; discrete plaques = +; no lysis = –.

The phage preparations were used in the test dilution with the exception of phage 6/12/19, which was used in a strength capable of producing confluent lysis of an hitherto untypable strain, strain 12. The phage types have been numbered only for the purpose of this table and are not recognized designations.

Twenty of the fifty strains were responsible for different outbreaks of food poisoning in human beings, and thirty were isolated from domestic animals. Phage typing did not serve to differentiate the animal strains from the human strains. No real opportunities have presented themselves to determine whether this method of typing would be of any value in studying the epidemiology of *S. thompson* infections. Three horses belonging to the same stud were found to be excreting the same phage type (type 9) of *S. thompson*, and three hens all from the same flock were also infected with an identical phage type (type 10). The faeces from a horse were examined several times over a period of 6 months and found to contain the same phage type (type 5) of *S. thompson* on each occasion.

The significance of lysogenic strains in phage typing

In a previous communication (Williams Smith, 1948) it was shown that acquired phage-resistance was responsible for many lysogenic strains of staphylococci being classified as different phage types. Table 1 shows that two strains (type 1) of *S. thompson* exhibited no evidence of acquired phage-resistance as they were fully susceptible to all the test phages. They were non-lysogenic. The remaining forty-eight strains showed some degree of resistance to one or more of the phages, and it was possible to show by repeated testing that forty-two of them were lysogenic. It was, therefore, considered possible that the phage resistance they had acquired, as indicated by the fact that they were lysogenic, may have been responsible for their resistance to some of the test phages. It was decided to investigate this possibility. The investigations were carried out in a manner similar to that which had been successful in elucidating the relationship among the staphylococcal strains. The principle of the method was to prepare a resistant variant of a strain (in this case a type 1 strain, strain 19), showing no evidence of acquired phage-resistance, to the phage or phages carried by a lysogenic strain that was resistant to one or more of the test phages. The resistant variant and the lysogenic strain were then tested with the test phages, and if they reacted in exactly the same manner it was concluded that acquired phage resistance was responsible for the lysogenic strain being considered to be a different phage type to type 1. The technical details of the method were as follows. A young broth culture of the lysogenic strain was maintained at 56° for 30 min. and then tested for bacterial sterility. Resistant variants of strain 19 were prepared to the 'crude phage' contained in the heat-treated culture. 'Crude phage' was used instead of purified phage as some strains were shown to be carrying more than one phage. The results of these experiments are shown in Table 2. The results obtained with the lysogenic strains 1, 2, 3, 14, 25, 47 and 288 and the resistant variants of strain 19 to the phages carried by these strains indicated that acquired phage-resistance accounted for these lysogenic strains being regarded as different phage types. Two types of resistant variants of strain 19 to the phage or phages carried by strain 288 were noted. One of them, 19 (288)*a*, behaved exactly the same as strain 288 and the other, 19 (288)*b*, was completely resistant to all the test phages. No evidence was obtained to show that acquired phage-resistance accounted for the classification of strains 5, 17, 49 and 7 as different phage types, although the resistant variants of strain 19 to the phages carried by these strains demonstrated that the phages they carried could account for the resistance of these strains to some, but not all, of the test phages. It may well be that the resistance exhibited by these strains had been acquired following infection with more than one phage in the field, and that they were now carrying only one phage; it had been shown (Williams Smith, 1951) that after strains

Table 2. *The action of the test phages on lysogenic strains and on variants of strain* 19 *resistant to the phages carried by these lysogenic strains*

	Phage preparations						
Strain	7/19	5/19	8/19	13/19	2/19	6/19	6/12/19
19	CL	CL	CL	CL	CL	CL	CL
1	CL	–	CL	–	CL	+	CL
19 (1)	CL	–	CL	–	CL	+	CL
2	–	–	–	–	–	CL	CL
19 (2)	–	–	–	+	–	CL	CL
14	–	–	–	–	–	CL	CL
19 (14)	–	–	–	–	–	CL	CL
3	–	–	CL	CL	CL	CL	CL
19 (3)	–	–	CL	CL	CL	CL	CL
25	–	–	CL	CL	CL	CL	CL
19 (25)	–	–	CL	CL	CL	CL	CL
47	CL	–	+	–	–	–	–
19 (47)	CL	–	+	–	–	–	–
288	–	–	–	CL	CL	CL	CL
19 (288)*a*	–	–	–	CL	CL	CL	CL
19 (288)*b*	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–
19 (5)	+	–	–	–	–	CL	CL
17	–	–	–	–	–	CL	CL
19 (17)	CL	–	CL	CL	CL	CL	CL
49	CL	–	–	+	–	CL	CL
19 (49)	CL	CL	CL	–	CL	CL	CL
7	–	–	–	–	–	–	CL
19 (17)	–	–	CL	CL	CL	CL	CL
8	–	CL	–	CL	CL	CL	CL
19 (8)	–	–	–	–	–	–	+

The resistant variants of strain 19 are designated by the number 19 followed by the number, in brackets, of the lysogenic strain from which the 'crude' phage was obtained.

All the phages, except 6/12/19, were used in test dilution.

Symbols as in Table 1.

of *S. thompson* were made resistant in the laboratory to two phages they frequently only carried one of them, the one to which they had first been made resistant. Alternatively, other phenomena may be responsible for the phage reactions of these four strains. The reactions of strain 19 (8), the variant of strain 19 made resistant to the phage carried by strain 8, were difficult to explain, since this variant was resistant to seven test phages, whereas strain 8 was only resistant to two of them. This resistant variant resembled strain 19 (288)*b*, since the induction of variants by bacteriophage action in both cases gave rise to strains which were resistant to all the test phages. Since both these variants were smooth, this change cannot be attributed to smooth-rough variation; but rather that phage action had induced variants lacking receptors for all phages and not only for the phage that had been used for their induction which, presumably, happens in most cases. These results question the validity of Bail's (1923) cross-resistance tests, since the essential part of these tests is that the variants, as far as phage resistance is concerned, should portray accurately the character of the phages which were responsible for their production. It was mentioned earlier that six of the forty-eight strains of *S. thompson* which showed some degree of resistance to the test phages could not be shown to be lysogenic, despite repeated attempts and, therefore, there was no evidence that they had acquired resistance as a result of infection in the field. It is well known that strains may be made resistant without subsequently being latently infected with phage, and this may have occurred in the case of these six strains.

That a lysogenic strain may carry two phages, both of which may have had some influence on the phage susceptibility of the strain was shown in the case of strain 14. The phage reactions of resistant variants of strain 19 to each of the two phages, *A* and *B*, carried by strain 14 and to both of these phages is shown in Table 3. Both phages were responsible in part for strain 14 being classified as a distinct phage type (type 9).

Table 3. *The action of the typing phages on variants of strain* 19 *resistant to the phages carried by strain* 14

	Phage preparations						
Strain	**7/19**	**5/19**	**8/19**	**13/19**	**2/19**	**6/19**	**6/12/19**
19	CL	CL	CL	CL	CL	CL	CL
19 (resistant to phage *A*)	–	–	CL	CL	CL	CL	CL
19 (resistant to phage *B*)	CL	+	–	–	–	CL	CL
19 (resistant to phages *A* and *B*)	–	–	–	–	–	CL	CL
14	–	–	–	–	–	CL	CL

Strain 14 was latently infected with two phages, *A* and *B*.
Symbols as in Table 1.

The effect of mouse passage on phage susceptibility

Table 1 shows that nine of the fifty strains of *S. thompson* were untypable. In view of the observation of Atkinson, Woodroofe, Macbeth, Chibnall & Mander (1949) that phage-resistant strains of *S. blegdam* could be rendered susceptible to the action of phages by mouse passage, six of the nine strains of *S. thompson* that were resistant to all the test phages were subjected to this procedure. They were then retested but were still found to be resistant. Atkinson *et al.* also noted that the virulence for mice of phage-susceptible strains of *S. blegdam* appeared greater than that of the resistant strains. No obvious difference was noted in the present work between the virulence of phage-resistant and phage-susceptible strains, although the numbers of mice used were too small to draw any definite conclusion.

The adaptation of phages to lyse untypable strains

The adaptation of phages to lyse bacteria previously considered untypable, by the selection and propagation of mutants, was first utilized by Craigie & Yen (1938) in the typing of *S. typhi*, and has since been used by other workers. Apparent mutation of one of the staphylococcal phages of Wilson & Atkinson

(1945) was shown by Rountree (1949) to be due to the phage preparation used for adaptation having been previously contaminated by another phage liberated during propagation on a lysogenic strain. This contaminating phage was liberated from the lysogenic strain. It was decided to investigate this phenomenon with regard to the *S. thompson* phages. A phage carried by strain 7 was propagated on the lysogenic strain 6. This phage 7/6 was 'purified' by plating with a culture of strain 6, picking single plaques and propagating three times. Attempts were now made to adapt 7/6 to lyse strain 14, a strain at one time regarded as untypable. Phage 7/6 was titrated on strains 14, 6 and 19 with the following results:

	Dilutions of phage 7/6				
Strain	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
14	++	+	—	—	—
6	CL	CL	CL	++	+
19	CL	CL	CL	++	+

A single plaque produced by the activity of phage 7/6 on strain 14 was picked, purified, and propagated on strain 14. The resulting phage preparation, 7/6/14, was found to be completely inactive on strain 6, its previous propagating strain. Further work showed that this phage behaved in exactly the same way as the phage carried by strain 6; cross-resistance tests showed these two phages to be completely identical. It was also shown that the phage carried by strain 7 after purification and propagation on the non-lysogenic strain 19, was without action on strain 14, and that it was quite impossible to isolate any mutants from this phage which were capable of lysing strain 14. It was, therefore, obvious that the apparent adaptation of phage 7/6 to lyse strain 14 was no more than the propagation on strain 14 of the phage carried by the lysogenic strain 6. This suggested that adequate precautions should be taken when studying phage mutation to ensure that phages are never propagated on lysogenic bacteria, since pure phage preparations cannot be obtained by this procedure; they will inevitably be contaminated by the phage carried by the lysogenic propagating strain.

The lytic action of the Salmonella thompson *phages on other species of salmonella*

The seven *S. thompson* phages, in the strength used for phage typing, were tested for activity on other species of salmonella. The species examined are listed below with the number of strains of each species placed in brackets.

GROUP A. *S. paratyphi* A (2).

GROUP B. *S. paratyphi* B (2), *typhi-murium* (4), *stanley* (1) *saint-paul* (1), *derby* (3), *california* (1), *abortus-equi* (1), *abortus-ovis* (2), *abortus-bovis* (2), *brancaster* (1).

GROUP C. *S. paratyphi* C (2), *cholerae-suis* (38), *cardiff* (1), *daytona* (1), *montevideo* (4), *oranianburg* (2), *potsdam* (10), *concord* (9), *barielly* (2), *tennessee* (2), *muenchen* (1), *manhattan* (1), *dusseldorf* (1).

Group D. *S. typhi* (2), *enteritidis* (2), *dublin* (3), *rostock* (2), *moscow* (1), *blegdam* (1), *pensacola* (1), *dar-es-salaam* (2), *gallinarum* (3), *pullorum* (3).

Group E. *S. london* (1), *give* (1), *anatum* (2), *meleagridis* (1), *zanzibar* (1).

Other groups. *S. habana* (1), *worthington* (1), *cubana* (1), *kirkee* (1), *cerro* (1), *kentucky* (1), *minnesota* (1), *inverness* (1).

The phages were completely inactive on all these strains except the one strain of *S. cardiff* and the *S. cholerae-suis* strains. The *S. cardiff* strain was susceptible to phage 6/12/19 only, this phage in reality being phage 6/19 used at a higher concentration than that necessary to produce confluent lysis of strain 19. Of the thirty-eight strains of *S. cholerae-suis*, thirty-two were smooth and six rough; sixteen were of the American and twenty-two of the *kunzendorf* type. Twenty-nine of the thirty-two smooth strains were susceptible to phages 8/19, 2/19, 6/19 and 6/12/19; one strain, C15, was also susceptible to phage 7/19. Two smooth strains and the six rough strains were resistant to all the phages. By using C15 as an indicator strain, twenty-three (88%) of the twenty-six smooth strains and five (83%) of the six rough strains were shown to be latently infected with the same phage, this phage being identical with phage 7/19 (strain C15 was non-lysogenic). It is probable that this fact would make a satisfactory classification of *S. cholerae-suis* difficult, since most of the strains would exhibit the same degree of phage resistance unless, of course, phages similar to those used by Craigie & Yen (1938) for typing *S. typhi* could be found.

DISCUSSION

A position appears to exist in *S. thompson* similar to that in staphylococci (Williams Smith, 1948). That is, acquired phage-resistance was responsible for many strains being classified as different phage types. Under laboratory conditions the phage types appeared to be stable in character. However, if a strain became infected with phage in the field it is probable that an alteration in its susceptibility to the test phages would result, and then the strain would be considered to be a different phage type. That such changes occurred in the field was shown in the case of staphylococci. It is obvious, too, that all lysogenic strains must have undergone at some time a change in phage susceptibility. The primary isolation of salmonella that are actively infected with phage is not a rare occurrence, and it is probable that these changes of type are not unusually rare. Faeces, too, are the natural habitat of salmonella and are a prolific source of different phages. It seems probable therefore that this method of phage typing could be usefully employed during a short-term investigation, such as that of an outbreak of food-poisoning due to *S. thompson*, but that the possibility of change of type may limit the usefulness of this method in investigations conducted over a long period of time. It has to be borne in mind that this method of typing probably classifies together strains with a similar history of phage infection and it would, therefore, be unprofitable to attempt to correlate this classification by phage with other phenomena such as virulence, species of animal host, or clinical symptoms.

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The Influence of Magnesium on Cell Division

4. The Specificity of Magnesium

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SUMMARY: Magnesium exerts a specific function in (*a*) the formation of the Gram complex, and the activation of cell division in certain Gram-positive bacteria, and (*b*) activation of bacterial growth. Of a series of divalent metallic ions, only manganese is capable of partially replacing magnesium in the above processes.

In previous communications (Webb, 1948, 1949*a*, *b*) magnesium was shown to be essential for the growth and cell division of the rod-shaped bacteria. In complex (i.e. peptone-water) media, conditions of magnesium deficiency or excess were found to inhibit the process of cell division, whereas in simple chemically-defined media, these conditions predominantly limited growth (i.e. synthesis of cell substance). The magnesium requirements of Gram-positive organisms in both simple chemically-defined media, and complex media were found to be considerably greater than the requirements of Gram-negative bacteria, and it was suggested that this difference may be due, in part at least, to the fact that the former contain magnesium as an essential component of the Gram-positive complex (cf. Henry & Stacey, 1946).

The scope of the work thus far recorded has been somewhat limited by the absence of a rapid and accurate gravimetric micro-method for the estimation of magnesium in bacterial cells and culture media. It appeared that the extension of these studies, as indicated elsewhere (Webb, 1949*c*, 1950), could be greatly facilitated by the replacement of magnesium by a functionally equivalent metallic ion for which a radio-active isotope could be used as a tracer. The magnesium isotope itself is unsuitable for this work owing to its extremely short half-life period.

The present paper describes the results of experiments in which other divalent metallic ions were examined for their ability to replace magnesium in the formation of the Gram complex, the activation of the processes of cell division in certain Gram-positive bacteria and the activation of cellular synthesis and metabolism.

EXPERIMENTAL

Most of the metallic ions studied had radii similar to that of the magnesium ion (Table 1) and, with the exception of manganese were used in the form of their sulphates or chlorides (Analar grade) without further purification. As the manganous and magnesium ions are isomorphous, manganese salts usually contain a relatively high percentage of magnesium. Pure manganous sulphate therefore was prepared from magnesium-free potassium permanganate by the following reactions:

(i) $2KMnO_4 + 3Na_2SO_3 + H_2O \rightarrow 2MnO_2 + 3Na_2SO_4 + 2KOH$;

(ii) $MnO_2 + SO_2 \rightarrow MnSO_4$.

The replacement of magnesium by other metallic ions was studied as follows.

Formation of the Gram complex

Washed cells from 3 l. of an 18 hr. culture of *Clostridium welchii* in 2 % (w/v) Evans's peptone water containing 0·5 % (w/v) glucose, were suspended in 2 % (w/v) sodium cholate (200 ml.) and kept at 60° until Gram-negative (16–18 hr.). These Gram-negative 'cytoskeletons' (Henry & Stacey, 1946) were collected by centrifuging, washed twice with water and suspended in 0·85 % (w/v) sodium chloride (100 ml., containing 2 % (v/v) of 40 % formaldehyde). After 16 hr. at room temperature the suspension was centrifuged, the deposit washed three times with water, and then suspended in a 2 % (w/v) solution of purified sodium ribonucleate (100 ml.; Henry & Stacey, 1946). Two ml. of this suspension were added to each of a series of test-tubes containing solutions (2 ml.) of the various metallic ions (Table 1), together with sodium ethyl mercurithiosalicylate (1:1000, 0·1 ml.). After 24 hr. at room temperature the approximate percentage of Gram-positive cells in each suspension (Table 1) was determined by the examination of stained smears.

Table 1. *The ability of other metallic ions to replace magnesium in the formation of the Gram complex of* Clostridium welchii

(Reduced Gram-negative cytoskeletons of *Cl. welchii* were suspended in 1% (w/v) sodium ribonucleate in the presence of the given metallic ion. Representative results below show the approximate percentage of Gram-positive cells observed in a series of stained smears after 18–24 hr. at room temperature.)

Metallic ion ($0·6 \times 10^{-3}$ g. ions/l.)	Ionic radius (A.)* according to Goldschmidt	Pauling	Gram-positive cells (% approx.)
Mg^{++}	0·78	0·65	80–100
Ca^{++}	0·99	—	10
Mn^{++}	0·91	0·80	80–100
Zn^{++}	0·83	0·74	0
Ni^{++}	0·78	0·69	0–10
Co^{++}	0·82	0·72	0
Fe^{++}	0·83	0·75	0
Be^{++}	0·34	0·31	—†
None (control)	—	—	0

* Taken from *Handbook of Chemistry and Physics* (30th ed. 1947, p. 2628), Chemical Rubber Publishing Co.

† There was complete precipitation of ribonucleic acid by beryllium. It will be shown elsewhere that beryllium resembles lanthanum in its ability to form highly insoluble salts with both ribo- and deoxyribonucleic acids. This property may have some significance with regard to the toxicity of beryllium.

Activation of cell division

A peptone medium deficient in magnesium was prepared as described previously (Webb, 1948). In this medium (L; Mg^{++} content *c.* 0·0001 %, w/v), Gram-positive bacilli and clostridia formed long filamentous cells. These filaments gave cells of normal morphology when subcultured in the same medium supplemented with 0·0015 % (w/v) magnesium (i.e. $6{\cdot}17 \times 10^{-4}$ g. ions/l.; medium M).

The ability of other divalent metallic ions to activate cell division was determined when inocula of filamentous cells from magnesium-deficient cultures (*Cl. welchii*, *Cl. tertium*, *Bacillus mycoides*, *B. vulgatus* and *B. subtilis* var. *viscosus*) were subcultured in medium M in which the Mg^{++} was replaced by other metallic ions at the same ionic concentration. When growth occurred, the cultures were centrifuged after 24–48 hr. at 37° and the morphological characteristics of the daughter cells determined in stained smears.

Under these conditions, the ions Co^{++}, Ni^{++}, Zn^{++} and Be^{++} did not replace Mg^{++} in the cell-division process. Moreover, these ions, even in the low concentrations employed, partially inhibited the growth of the organisms studied, the toxicity of the ions increasing in the above order. The filamentous cells observed in cultures of both the clostridia and bacilli containing Zn^{++} and Co^{++} were considerably longer than those observed in control cultures of the same organisms in medium L. The addition of these ions to medium M, however, failed to produce filaments. Thus, although Zn^{++} and Co^{++} are unable to replace Mg^{++} in cell division, it appears possible that, because of their similarity to the magnesium ion in valency and ionic radius, they may exhibit a type of competitive antagonism and prevent the uptake by the growing organisms of the residual Mg^{++} in the Mg^{++} deficient medium.

The growth of *B. subtilis* var. *viscosus*, *B. mycoides* and *B. vulgatus* was stimulated by Mn^{++} almost to the same extent as by Mg^{++} at the same concentration. The cells from these cultures varied in morphology from almost coccoidal forms to normal rods. Filamentous cells, however, were not observed.

With *Cl. welchii* and *Cl. tertium* the addition of Mn^{++} to the medium increased the amount of growth more than the addition of Mg^{++}. However, whereas the cells from the cultures containing the latter were of normal morphology, those from cultures containing Mn^{++} were filaments, and appeared essentially similar to those observed in the control cultures (medium L).

From these results it appeared that in the anaerobic cultures the divalent manganous ion was oxidized to the tri- or tetravalent state. This oxidation might conceivably enhance the growth of the anaerobes by maintaining reducing conditions, but, at the same time, would convert the manganese into a form in which it no longer resembled the magnesium ion. Accordingly, medium L (20 ml.) was supplemented by the addition of reducing agents (thiolacetic acid, 0·1 ml.; cysteine hydrochloride, 2 mg.; or L-ascorbic acid, 2 mg.). Although under these conditions, the stimulation of growth of either *Cl. welchii* or *Cl. tertium* which followed the addition of Mn^{++} to the medium was less than that which occurred when Mg^{++} was added, the final population was composed of short rods of normal morphology and filamentous cells were not observed.

Activation of growth in a simple chemically-defined medium

Of a number of chemically-defined media studied, that described by Koser & Rettger (1919), when containing 40 p.p.m. magnesium, supported the growth of all the organisms examined (*B. vulgatus*, *B. mycoides*; *Aerobacter cloacae*, *Escherichia coli commune* and *Pseudomonas aeruginosa*) and appeared most suitable for comparative purposes. The medium was prepared free from Mg^{++}, as previously described (Webb, 1949*b*). The ability of other metallic ions to initiate growth in this medium was determined over the concentration ranges of 0 to $4{\cdot}12 \times 10^{-4}$ g. ion/l. (Fig. 2) and 0 to $4{\cdot}12 \times 10^{-3}$ g. ion/l. (Fig. 1); these ranges are equivalent to 0–10 and 0–100 p.p.m. Mg^{++} respectively. Under these conditions, no growth of the above organisms occurred in the presence of Ni^{++}, Co^{++}, Zn^{++}, Be^{++} or Fe^{++}. Some growth occurred in the presence of Mn^{++}, but even this metal was somewhat toxic in the higher concentrations (cf. Gwan, 1948), and failed to produce an activation of growth comparable to that produced by Mg^{++}.

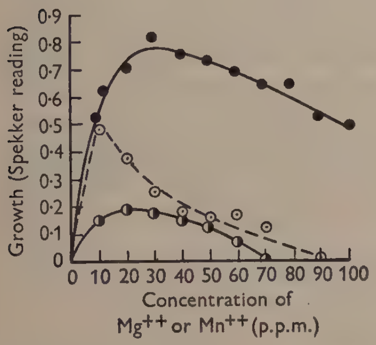

Fig. 1

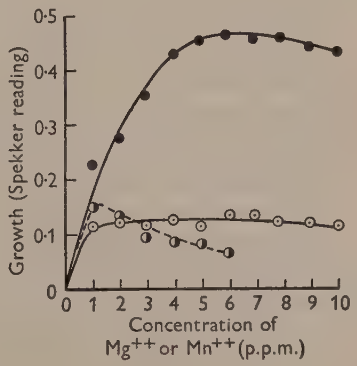

Fig. 2

Figs. 1 and 2. Influence of magnesium and of manganese concentration on the growth of certain Gram-positive and Gram-negative bacteria in a chemically-defined medium (Koser & Rettger, 1919) free from these ions. Growth was measured turbidimetrically with the Spekker photoelectric absorptiometer as previously described (Webb, 1949*b*).

Fig. 1. Effect of magnesium concentration (●——●) and manganese concentration (◑——◑) on the growth of *B. mycoides*. (⊙——⊙), effect of the manganese concentration of the medium on the growth of *B. vulgatus*.

Fig. 2. Effect of magnesium concentration (●——●) and manganese concentration (◉——◉) on the growth of *A. cloacae*. (◑——◑), effect of the manganese concentration of the medium on the growth of *Esch. coli commune*.

DISCUSSION

The ability of Mn^{++} to replace Mg^{++} in the formation of the Gram complex and, under certain conditions, in the activation of cell division is in accordance with other findings in biological systems. Thus, as is well known, many enzymes which require Mg^{++} for activation may also be activated by Mn^{++} (e.g. Nilsson, Alm & Burstrom, 1942). However, it appears from the results given in Figs. 1 and 2 that in certain of the Mg^{++}-activated enzyme reactions associated with growth and metabolism the function of magnesium cannot be replaced by manganese. In this connexion, it is of interest to recall that Quastel & Webley (1942) found that Mn^{++} could not replace Mg^{++} in the activation of the oxidation of acetate by certain bacteria which required vitamin B_1 as a nutrient.

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The Influence of Magnesium on Cell Division

5. The Effect of Magnesium on the Growth of Bacteria in Chemically-Defined Media of Varying Complexity

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SUMMARY: The growth of bacteria in chemically-defined media containing mixtures of amino-acids is less dependent upon the magnesium concentration than in simple media composed of salts, glucose or glycerol and a single source of nitrogen. In the more complex chemically-defined media growth initially increases rapidly with, and then becomes independent of, the magnesium concentration. Gram-positive bacilli fail to grow when the magnesium content of the medium is less than 0·66 p.p.m., whereas this amount of magnesium is almost sufficient to maintain maximum growth of the Gram-negative bacteria. In addition, a deficiency of magnesium (i.e. *c.* 1–5 p.p.m.) inhibits the cell division of the Gram-positive rod-shaped organisms, and results in the formation of filamentous cells.

The importance of ionic magnesium in the processes of bacterial growth and cell division has been recorded in previous papers (Webb, 1948, 1949*a*, *b*, 1951). In peptone media, a deficiency of magnesium predominantly inhibits the cell division of Gram-positive rods and results in the formation of filamentous cells. In simple chemically-defined media, however, the growth of bacteria is a function of the magnesium concentration, and in such media containing suboptimal amounts of magnesium, filamentous cells are not observed.

To explain these differences it was suggested (Webb, 1949*b*) that magnesium is essential both for the synthesis of intermediate metabolites and for cell division. In complex media which already contain some or all of the intermediate metabolites, the synthetic activities of the cell are decreased and a deficiency of magnesium inhibits cell division. However, in simple chemically-defined media, where synthesis precedes cell division, a deficiency of magnesium inhibits growth and normal cell division occurs throughout the restricted population that the medium is able to maintain.

Evidence in favour of the above hypothesis is provided by the present communication which records the results of an investigation of the effect of magnesium on the growth of bacteria in more complex chemically-defined media containing amino-acids and other growth factors. Under these conditions, the growth of bacteria is less dependent upon the magnesium concentration, and a deficiency of magnesium primarily inhibits the cell division of the Gram-positive rods.

MATERIALS AND METHODS

It was found in early experiments that in media containing several amino-acids but no added magnesium, the cumulative effect of traces of magnesium in the commercial amino-acids used was sufficient to maintain the growth of bacteria for an indefinite number of subcultures. Similar results were obtained after treatment of concentrated solutions of the amino-acids at pH 9 with

8-hydroxyquinoline according to the method of Waring & Werkman (1942); the magnesium content of the media was then of the order of 1–2 p.p.m. when estimated by comparative methods using Titan yellow. Media 'biologically-free' from magnesium were finally obtained by a modification of the method of Nicholas (1951, and personal communication).

Pyrex glassware, used throughout, was cleaned in concentrated chromic-sulphuric acid mixture rinsed in distilled water, 0·1 M sodium bicarbonate (Analar) in glass-distilled water, 0·1 N nitric acid (redistilled) in double-distilled water, and washed in six changes of double-distilled water. The glassware was dried at 180° in Pyrex containers. Spatulas and Pasteur pipettes were made from Pyrex glass rod and tubing, respectively, cleaned as above.

A concentrated solution of the nutrient substances, other than glucose, phosphate and the micro-nutrients, in double-distilled water, was adjusted to pH 9 with ammonium hydroxide (prepared by collecting ammonia in double-distilled water) and 5 ml. of a 2 % (w/v) solution of 8-hydroxyquinoline in 2 N acetic acid (Analar) added. After 24 hr. at 37° the solution was filtered through Whatman no. 5 filter-paper, and the filtrate extracted six times with redistilled chloroform to remove excess 8-hydroxyquinoline and then six times with redistilled ether to remove residual chloroform. Micro-nutrients (Fe^{+++}, Cu^{++}, as required) and glucose were added, the solution diluted to volume with double-distilled water, adjusted to pH 6·5 with a standard solution of constant boiling hydrochloric acid in double-distilled water and sterilized by steaming.

As glucose was decomposed with the formation of toxic products at the alkaline pH used in the above purification, trace metals were removed from a concentrated solution of the sugar by a column of ion exchange resin (Zeo-karb 215). The column was prepared as follows:

A suspension of Zeo-karb 215 in 0·1 N hydrochloric acid (concentrated hydrochloric acid distilled in a Pyrex still, and the constant-boiling distillate diluted with double-distilled water) was introduced into a Pyrex glass tube (30 × 1 cm.) cleaned in the usual way, and containing a plug of filter-paper previously extracted with 0·1 N hydrochloric acid, at a constriction 4 cm. from the lower end. The column was washed with double-distilled water (500 ml.), and then a solution of glucose (25 g.) in double-distilled water (250 ml.) allowed to run slowly through the resin. The first 50 ml. of effluent were discarded; the remainder, containing the bulk of the glucose, was adjusted to *c.* pH 6·0 with ammonium hydroxide and sterilized. Analyses for reducing sugar by the method of Shaffer & Hartman (1920) showed that no loss of glucose occurred. Control experiments, in which glucose solutions containing varying concentrations of magnesium sulphate were passed through such columns and then analysed qualitatively with Titan yellow, showed that the magnesium was removed completely.

Phosphate solutions adjusted to pH 6·5 with ammonium hydroxide, were sterilized separately in steam and added to the main bulk of the medium when cold. The potassium dihydrogen phosphate (Analar) used was recrystallized four times from a mixture of double-distilled water and twice-distilled ethanol, and dried at 80°.

EXPERIMENTAL

Influence of magnesium on bacterial growth in complex chemically-defined media

Preparation of inoculum. The technique used to obtain inocula free from magnesium was as previously described (Webb, 1949*b*). Subcultures were made from stock into the chemically defined media and incubated for 48 hr. Serial subcultures were then made into the same medium every 48 hr. until the final subculture failed to grow. The penultimate culture was used as inoculum. It was necessary to subculture Gram-negative bacteria about six times before growth ceased, whereas two or three subcultures sufficed for the Gram-positive organisms (Table 1). These results already indicate a difference in the magnesium requirements of these two types of organisms.

Table 1. *Relative amounts of growth in serial subcultures of Gram-positive and Gram-negative organisms in Heigener's* (1935) *medium, free from magnesium*

	Growth (Spekker reading) after 48 hr. at 37°					
Subculture no.	*Serratia marcescens*	*Aerobacter aerogenes*	*Aerobacter cloacae*	*Bacillus mesentericus*	*Bacillus mycoides*	*B. subtilis* var. *viscosus*
1	0·352	0·333	0·340	0·098	0·075	0·076
2	0·283	0·212	0·272	0·032	0·040	0·049
3	0·212	0·193	0·218	0·000	0·000	0·002
4	0·003	0·154	0·075	—	—	—
5	—	0·080	0·005	—	—	—
6	—	0·003	0·000	—	—	—

An increase in polysaccharide synthesis was observed in the serial subcultures of the organisms listed in Table 1. This effect of magnesium deficiency was particularly marked with the Gram-negative organisms; cultures of *Pseudomonas aeruginosa*, for example, became so viscous that the growth could not be measured turbidimetrically.

Chemically-defined media. The media used (Table 2) varied from a simple mixture of four amino-acids, pyruvic acid, nicotinic acid and mineral salts (medium 1) to the complex solutions required for the nutrition of the lactobacilli, streptococci, *Kurthia zenkeri* (medium 4) and *Clostridium welchii* (medium 5). The influence of magnesium on the growth of various Gram-positive organisms in these nutrient solutions is shown in Figs. 1 and 2 and Table 3. When these results are compared with those previously obtained with the same organisms grown in simple chemically-defined media (Webb, 1949*b*), it is apparent that the growth of Gram-positive bacteria in the presence of amino-acids and other growth factors is much less dependent upon the magnesium concentration of the medium (cf. Fig. 5 and also Figs. 1 and 2 of the preceding paper (Webb, 1951)).

Growth of the lactobacilli and streptococci in medium 4 (Fig. 2) occurred at zero magnesium concentration, since this medium contained a low concentration of manganese (2 p.p.m.), and, as previously shown (Webb, 1951), this metal can partially replace magnesium in bacterial growth and cell division.

Table 2. *The organic composition of the chemically-defined media used*

(Composition: quantities shown per 100 ml. medium.)

Medium 1. L-Asparagine, L-cysteine HCl, L-glutamic acid and L-arginine HCl (50 mg.), nicotinic acid (1 mg.), pyruvic acid (0·5 g.)

Medium 2. (Heigener, 1935.) DL-Valine (0·234 g.), glycine (0·150 g.), DL-alanine (0·178 g.), DL-phenylalanine (0·33 g.), DL-leucine (0·262 g.), L-asparagine (0·264 g.), L-glutamic acid (0·294 g.), glucose (0·250 g.)

Medium 3. (Glinka-Tschernorutzky, 1933.) Glycine (50 mg.), DL-valine (50 mg.), DL-alanine (50 mg.), DL-phenylalanine (50 mg.), DL-leucine (50 mg.), L-glutamic acid (50 mg.), DL-aspartic acid (50 mg.), L-arginine (50 mg.), L-cystine (25 mg.), L-tryptophan (25 mg.), DL-tyrosine (25 mg.), L-proline (25 mg.), L-histidine HCl (25 mg.), DL-lysine HCl (25 mg.), DL-serine (25 mg.), glucose (0·5 g.)

Medium 4. (Krueger & Peterson, 1945, modified.) Amino-acid mixture as in medium 3, with addition of DL-isoleucine (25 mg.), DL-norleucine (25 mg.), DL-threonine (25 mg.), DL-methionine (25 mg.), L-cysteine HCl (5 mg.), adenine sulphate (3·0 mg.), guanine hydrochloride (2·0 mg.), riboflavin (25 μg.), calcium-D-pantothenate (50 μg.), nicotinic acid (250 μg.), pyridoxin hydrochloride (50 μg.), *p*-aminobenzoic acid (25 μg.), aneurin (5 μg.), biotin (0·001 μg.), folic acid (0·001 μg.), sodium acetate (0·6 g.), glucose (1·0 g.)

Medium 5. (Boyd, Logan & Tytell, 1948, modified.) Amino-acid mixture as in medium 3 with omission of DL-alanine and DL-lysine, and addition of DL-methionine (5 mg.), L-cysteine HCl (5 mg.), adenine sulphate (3 mg.), guanine hydrochloride (2·0 mg.), uracil (1·7 mg.), biotin (0·05 μg.), calcium-D-pantothenate (100 μg.), pyridoxin (50 μg.), riboflavin (50 μg.), ascorbic acid (25 mg.), glucose (0·5 g.), ammonium lactate (0·5 g.).

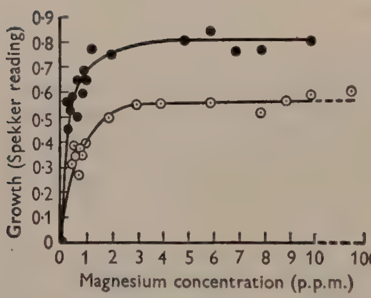


Fig. 1

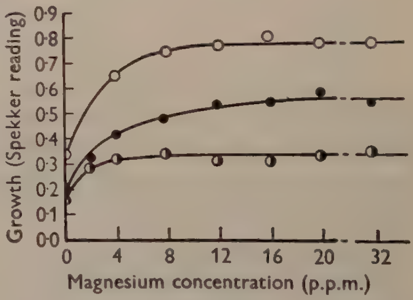


Fig. 2

Fig. 1. The influence of magnesium on the growth of *Bacillus vulgatus* (●——●) and *B. mycoides* (⊙——⊙) in Glinka-Tschernorutzky (1933) medium (medium 3, Table 2). Growth measured turbidimetrically after 72 hr. at 37°.

Fig. 2. The influence of magnesium on the growth of *Lb. casei* (○——○), *Strep. pyogenes* (●——●) and *Strep. faecalis* (◑——◑) in medium 4, Table 2 containing 2 p.p.m. Mn^{++}. Growth measured turbidimetrically after 80 hr. at 37°. Growth curves similar to the above were obtained with *Lb. delbrueckii* and *K. zenkeri* and are not shown in the figure.

Although no growth occurred in the complete absence of magnesium and manganese (Figs. 1, 2; Table 3), maximum growth was attained when the magnesium concentration of the medium was 2–3 p.p.m. Above this value the amount of growth was independent of further increases in the magnesium concentration (up to 100 p.p.m.). These results contrast with those obtained with simple chemically-defined media containing only salts, glucose or glycerol and a suitable source of nitrogen (Webb, 1949*b*) where concentrations of magnesium much above the optimal inhibited the growth of a number of species of bacteria. It appears that in the complex chemically-defined media the presence of the amino-acids decreases the inhibitory or toxic effect of magnesium ions.

Table 3. *Effect of magnesium concentration on the growth of* Bacillus polymyxa, B. subtilis *and* Clostridium welchii *in chemically-defined media*

Magnesium concentration (p.p.m.)	Relative growth		
	*B. polymyxa**	*B. subtilis**	*Cl. welchii*†
0·0	0·00	0·00	0·00
1·0	0·575	0·289	0·350
2·0	0·610	0·320	0·372
3·0	0·630	0·350	0·405
4·0	0·649	0·350	0·335
5·0	0·592	0·358	0·373
6·0	0·620	0·332	0·375
7·0	0·600	0·352	0·374
8·0	0·628	0·356	0·365
9·0	0·608	0·361	0·385
10·0	0·622	0·331	0·370

* Growth measured turbidimetrically (Spekker absorptiometer) after 48 hr. at 37° in medium 1 (Table 2).

† Growth (in medium 5, Table 2) measured turbidimetrically after 24 hr. at 37° in an atmosphere of hydrogen.

A closer study was made with both Gram-positive and Gram-negative organisms at very low concentrations of magnesium (0–1·8 and 0–0·8 p.p.m. respectively). No growth of the Gram-positive organisms occurred when the magnesium concentration of the medium was less than 0·66 p.p.m. (Fig. 3), whereas this concentration was almost sufficient to maintain the maximum growth of the Gram-negative bacteria (Fig. 4). These results (Figs. 3, 4), however, show also that in addition to magnesium, the organic constituents of the medium influence the growth of the various bacteria, and suggest that the true influence of magnesium on bacterial growth under these conditions can be determined only when each organism is grown in a medium defined to suit its own nutritional requirements.

It was observed that growth occurred initially in the culture containing the highest concentration of magnesium. This effect of magnesium on the lag phase of the growth of *Bacillus mycoides* in medium 1 is shown in Table 4. The turbidimetric measurements recorded in Table 4 were made on growing cultures, wooden adaptors being constructed to hold the test-tubes in the cell mount of the Spekker absorptiometer. In consequence, although the tubes selected for the cultures appeared uniform, it is probable that the results of Table 4 represent a qualitative, rather than quantitative, expression of the amount of growth.

The influence of magnesium concentration on morphology

Gram-positive bacteria. Filamentous cells (Pl. 1, figs. 1–3) were observed consistently in stained smears prepared throughout the serial subcultures of the Gram-positive rods recorded in Table 1, and in the assay tubes containing the lower magnesium concentrations (Figs. 1, 2; Table 3). Organisms grown in Glinka-Tschernorutzky's (1933) complex medium (medium 3, Table 2; Fig. 1) under conditions of magnesium deficiency, formed extremely long filaments, many of which extended over several fields of the microscope (Pl. 1, figs. 4, 5).

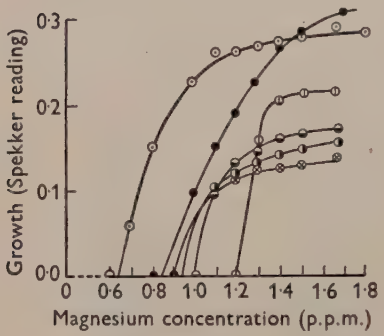


Fig. 3

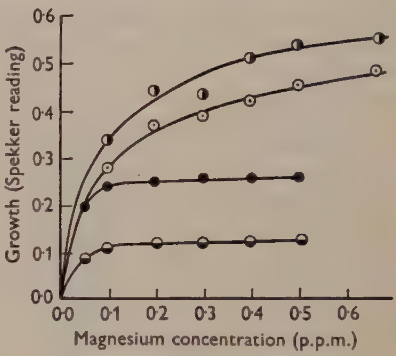


Fig. 4

Fig. 3. The influence of low concentrations of magnesium on the growth of certain Gram-positive bacilli. *B. subtilis* var. *viscosus* (⊙——⊙), *B. mesentericus* (⦶——⦶), and *B. mycoides* (◒——◒) grown in medium 1, Table 2; *B. subtilis* var. *viscosus* (⊗——⊗), *B. mesentericus* (◑——◑) and *B. mycoides* (●——●) grown in medium 2, Table 2. Growth measured turbidimetrically after 72 hr. at 37°.

Fig. 4. The influence of low concentrations of magnesium on the growth of certain Gram-negative bacteria. *A. aerogenes* (◑——◑), and *A. cloacae* (⊙——⊙) in medium 2, Table 2. *A. aerogenes* (●——●) and *Serratia marcescens* (◒——◒) in medium 1, Table 2. Growth measured turbidimetrically after 72 hr. at 37°.

Table 4. *Effect of magnesium concentration on the lag phase of the growth of* Bacillus mycoides *in medium* 1 (*Table* 2) *at* 37°

	Magnesium concentration (p.p.m.)			
	1·1	1·2	1·3	1·4
Time (hr.)	Relative growth (Spekker reading)			
0	0·000	0·000	0·000	0·000
12	0·000	0·000	0·000	0·090
24	0·000	0·040	0·101	0·134
36	0·000	0·090	0·101	0·160
48	0·000	0·101	0·144	0·170
72	0·095	0·138	0·148	0·164

With increasing concentrations of magnesium there was a gradual change in the morphology of the cells, roughly as follows:

Morphology:	Filaments →		Shorter filaments →	Filaments and chains →	Chains and 'normal' rods →	Isolated 'normal' rods
Magnesium concentration (p.p.m.)	0	4	10	12	24	28 and greater

The above values are approximate only, and considerable overlapping of these limits occurred in many experiments. However, it may be of some significance that an abrupt transition from filaments to 'normal' rods was not observed.

Filament formation occurred also in magnesium-deficient cultures of *Lactobacillus casei*, *Lb. delbrueckii* and *Kurthia zenkeri* in medium 4, although the incidence of cells of abnormal length was decreased by the presence of the manganese ions. Cultures of the strains of staphylococci which grew in this medium contained an increased percentage of oval and giant cells at low magnesium concentrations. No change in the morphology of either *Streptococcus pyogenes*, or *Strep. faecalis* was observed when these organisms were cultivated in medium 4 containing 0–4 p.p.m. of magnesium.

Gram-negative rods. No gross change in morphology was observed in cultures of *Aerobacter aerogenes*, *Serratia marcescens* and *Pseudomonas aeruginosa* under the conditions of the experiments of Fig. 4. A small percentage of short filaments was observed in cultures of *A. cloacae*, *Escherichia coli* var. *acidilactici* and *Esch. coli commune* (cf. Pl. 1, fig. 6) in either medium 1 or medium 2 (Table 2) containing low concentrations of magnesium (0·1–0·7 p.p.m.).

Influence of the amino-acid composition of a magnesium-deficient medium on the morphology of Gram-positive cells

The effect of different magnesium concentrations on the growth of *B. mycoides* in a simple chemically-defined medium (Koser & Rettger, 1919), and in the same medium, but containing in addition, L-glutamic acid (0·05 % w/v), L-tryptophan (0·05 % w/v), L-cystine (0·05 % w/v), L-arginine (0·05 % w/v) and nicotinic acid (0·001 % w/v), is shown in Fig. 5. In the simple medium (medium A), growth was a function of the magnesium concentration and reached a maximum at *c.* 40 p.p.m., whereas in the medium supplemented with amino-acids and nicotinic acid (medium B), growth rapidly became independent of changes in magnesium concentration. Furthermore, normal cell division occurred in all cultures of *B. mycoides* in medium A irrespective of the magnesium concentration, whereas in medium B filamentous cells were observed in cultures containing 5 p.p.m. of magnesium. It thus appeared possible that the synthetic processes, which, in medium A, required magnesium for activation, were those concerned with the formation of the amino-acids present in medium B. In an attempt to determine whether magnesium was associated with the synthesis of one or all of the amino-acids (or nicotinic acid) each was added in turn to the basal medium (Koser & Rettger, 1919) containing 5 p.p.m. of magnesium. Under these conditions it was to be expected that filamentous cells would be observed in the culture containing the amino-acids which, in the simple basal medium, was synthesized by a magnesium activated enzyme system. Each of the above substances stimulated the growth of *B. mycoides*, the greatest increase in growth occurring in the presence of glutamic acid (Table 5). Filamentous cells, however, were not observed in any of these cultures.

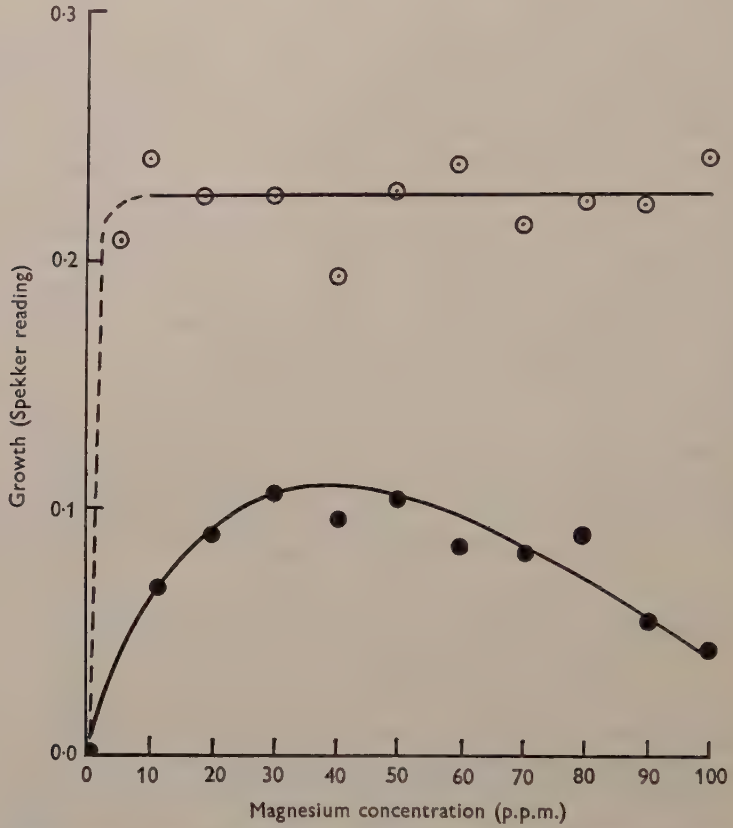


Fig. 5. The influence of magnesium on the growth of *B. mycoides* in Koser & Rettger's (1919) medium (●——●), and in the same medium supplemented by the addition of L-glutamic acid (0·05 %), L-tryptophan (0·05 %), L-cystine (0·05 %), L-arginine (0·05 %) and nicotinic acid (0·001 %) (⊙——⊙).

In further experiments, the influence of various combinations of these amino-acids on the growth and cell division of *B. mycoides* was determined. Although filaments were formed in cultures containing only these four amino-acids, variable results were obtained when each was omitted in turn. Similar experiments, in which other combinations of amino-acids were studied, suggested that a certain minimal number of amino-acids (*c.* 4–6, according to the constitution of the individual amino-acids) was necessary before a deficiency of magnesium resulted in the formation of filamentous cells. In this connexion it is of interest to note that Stephens & Hinshelwood (1949) showed that each addition of an amino-acid to a simple chemically-defined medium resulted in a progressive shortening of the mean generation time of *A. aerogenes* until, when some twenty amino-acids were present, the optimal mean generation time was attained.

Table 5. *Effect of certain amino-acids and nicotinic acid on the growth of* Bacillus mycoides *in Koser & Rettger's* (1919) *medium containing* 5 *p.p.m. of magnesium*

(The table summarizes the results of four independent experiments.)

Basal medium supplemented by	Relative growth (Spekker reading) 24 hr.	48 hr.
—	0·030	0·075
L-Glutamic acid	0·119 0·124 0·126	0·325
L-Arginine	0·056 0·051 0·049	0·263
L-Cystine	0·045 0·048 0·058	0·126
L-Tryptophan	0·048 0·030 0·031	0·195
Nicotinic acid	0·042 0·045 0·045	0·121

Furthermore, Gale (1949) has shown that either magnesium or manganese, or both, are involved in the assimilation of glutamic acid, and presumably certain other amino-acids, by Gram-positive cells (*Staph. aureus*). Since the co-ordination complex of 8-hydroxyquinoline with manganese is more stable than the corresponding complex with magnesium, it is apparent that a medium rendered biologically free from magnesium with 8-hydroxyquinoline, must also be free from manganese. Thus, the assimilation of certain amino-acids from the chemically-defined media (other than medium 4) used in the present work must be activated by magnesium alone. In addition, there appears to be a certain parallel between the effect of magnesium on the growth of Gram-positive bacteria and its effect on the assimilation of amino-acids. For example, the assimilation of glutamic acid by *Staph. aureus* cells *deficient* in magnesium and manganese, initially increases rapidly with increases in the concentration of added magnesium, reaches a maximum at *c.* 10^{-5} M (0·26 p.p.m.) and then becomes independent of further increases in the magnesium concentration up to 10^{-2} M, $=260$ p.p.m. (Gale, 1949, Fig. 5). Similarly, the growth of Gram-positive bacteria increases rapidly with increasing magnesium concentration, reaches a maximum at 2–4 p.p.m. and then becomes independent of further increases in the magnesium concentration (Figs. 1, 2, Table 3).

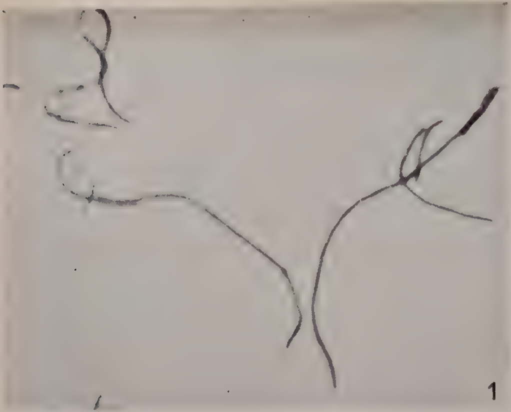
DISCUSSION

In a simple chemically-defined medium containing salts, glucose or glycerol, and a source of nitrogen, the magnesium requirements of Gram-positive organisms are about ten times as great as those of Gram-negative bacteria (Webb, 1949*b*). The results described in this paper also show that even in complex chemically-defined media, Gram-positive bacteria require more magnesium for maximum growth than do the Gram-negative species. In accordance with these facts, Gram-positive cells harvested from a culture medium of constant composition appear to contain greater amounts of magnesium than do Gram-negative organisms grown in the same medium (Webb, 1949*a*).

However, it is clear that much of the magnesium of Gram-positive bacteria (e.g. *c.* 70 % in the case of *Cl. welchii*) is co-ordinated into the structure of the Gram complex (Henry & Stacey, 1946) and is not available for the activation of essential systems of the cell. Thus it is probable that comparable concentrations of magnesium are required by both Gram-positive and Gram-negative organisms under normal conditions of growth for the activation of certain enzyme mechanisms such as those involved in the energy-yielding reactions, synthetic processes, assimilation and cell division. In simple chemically-defined media, where the synthetic activities of the cell are at a maximum, the concentration of magnesium necessary for the growth of a given organism is greater than that in a complex chemically-defined medium containing a range of essential metabolites. In media of the latter type, where the synthetic activities of the cell are decreased, low concentrations of magnesium produce little effect upon growth. In the case of the Gram-positive rods, however, these conditions are insufficient to maintain normal cell division and, in consequence, filamentous cells are formed.

Although much of the work recorded in this and previous papers has been carried out with strains of bacilli and clostridia, the fact that low concentrations of magnesium induce the formation of filaments in cultures of lactobacilli and *Kurthia* indicates that the effect of magnesium on cell division is not confined specifically to the Bacillaceae. At present it is not clear why a deficiency of magnesium in complex chemically-defined media inhibits the cell division only of the Gram-positive rods. It seems unlikely that Gram-positive and Gram-negative bacteria divide by different mechanisms, or that magnesium is essential for the cell division of the former, and not the latter. Gale and associates (e.g. Gale 1947, 1948) have shown that there is a marked difference between the amino-acid metabolism of Gram-positive and Gram-negative bacteria. It is possible, therefore, that the apparent lack of effect of magnesium deficiency on the morphology of Gram-negative rods is linked in some way with the metabolism of these cells in complex amino-acid mixtures.

Thanks are due to Prof. M. Stacey, F.R.S., and Dr H. B. Fell for their interest in this work, and to Messrs Z. Hybs, B.Sc., and G. F. Davenall for assistance with certain of the preliminary experiments which were carried out at the University of Birmingham. Grateful acknowledgement is made to Dr D. J. D. Nicholas for his kindness in making available the details of his 8-hydroxyquinoline method of purification prior to publication.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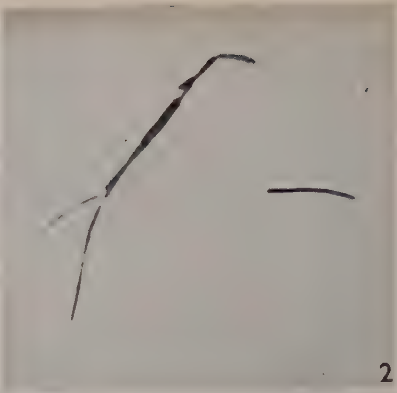
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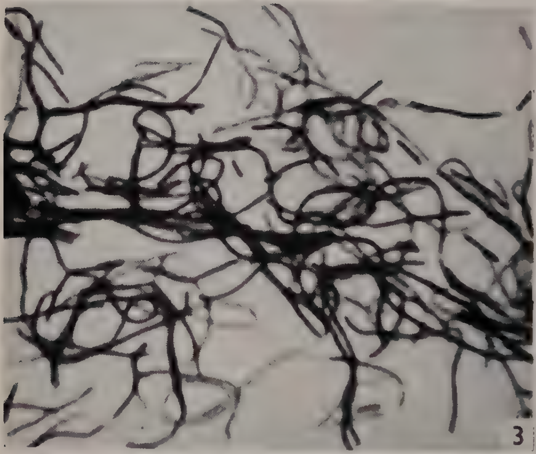
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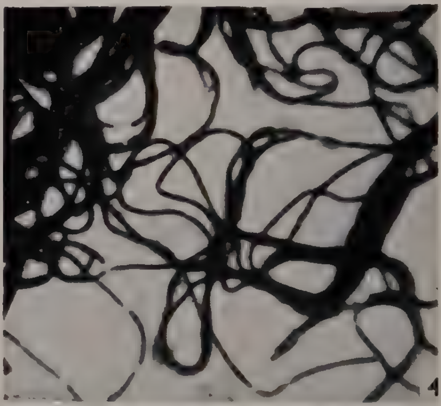
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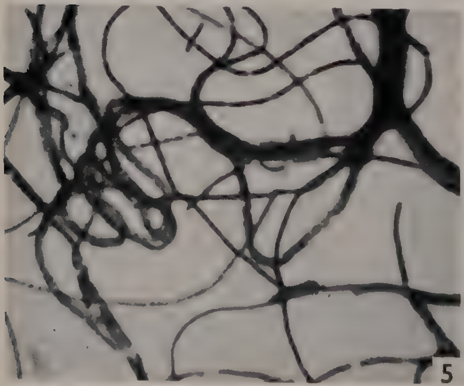
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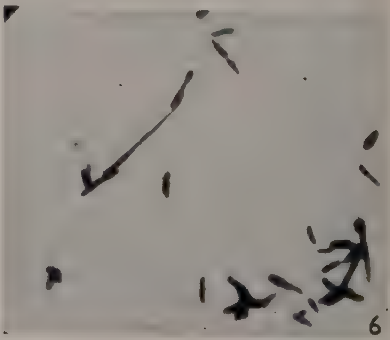
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Figs. 1–6

M. WEBB—MG AND BACTERIAL GROWTH IN AMINO-ACID MEDIA. PLATE 1

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EXPLANATION OF PLATE

(Magnification: figs. 1–5, ×1000; fig. 6, ×1400.)

Fig. 1. Filamentous forms of *Bacillus subtilis*, grown on Heigener's (1935) medium (medium 2, Table 2) containing 2 p.p.m. Mg^{++}.

Fig. 2. *B. mycoides* after one subculture in Heigener's (1935) medium in the absence of magnesium (cf. Table 1).

Fig. 3. *Clostridium welchii* grown in medium 5 (Table 2) containing 5 p.p.m. Mg^{++}.

Figs. 4, 5. *B. subtilis* (fig. 4) and *B. mycoides* (fig. 5) grown in a complex amino-acid mixture (Glinka-Tschernorutzky, 1933) containing 2 p.p.m. Mg^{++}.

Fig. 6. *Escherichia coli commune* grown in Heigener's (1935) medium containing 0·3 p.p.m. Mg^{++}.

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The Influence of Magnesium on Cell Division

6. The Action of Certain Hydrolytic Enzymes on the Filamentous and Chain Forms of Gram-Positive Rod-Shaped Organisms

BY M. WEBB

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SUMMARY: The chains which are formed when rod-shaped Gram-positive bacteria are grown in media containing low concentrations of magnesium, undergo fission when exposed to the action of lysozyme. This effect is not produced by various other hydrolytic enzymes including the carbohydrases, cellulase and hyaluronidase. Fragmentation of the filamentous forms of the Gram-positive organisms, however, is not produced by lysozyme.

A crude preparation obtained from mechanically disintegrated *Clostridium welchii* cells resembled lysozyme in its action on the magnesium-deficient organisms.

The growth of Gram-positive rod-shaped bacteria in complex media (peptone water or amino-acid mixtures) deficient in magnesium, results in the inhibition of cell division and in the formation of filamentous cells (Webb, 1948*a*, 1949, 1951). Since magnesium deficiency does not affect the division of the nuclear structures (chromatinic bodies) and, with increasing concentrations of magnesium, the morphology of the cells changes from filaments to chains and thence to isolated normal rods (Webb, 1949), it appears probable that magnesium is involved in one of the final stages of cell division.

From these considerations it was of interest to determine whether, by the action of hydrolytic enzymes of known specificity, it was possible to cause fragmentation of the filaments or the chains. Since little was known of the chemical nature of the cellular components affected by magnesium deficiency, a study was made of the action of various groups of enzymes, each of which exhibited hydrolytic activity against particular macromolecular constituents of the cells.

MATERIALS AND METHODS

Source of enzymes

The enzymes used in the following experiments are summarized below. The pH value at which the action of a given enzyme was studied and its concentration (mg./5 ml.) in a mixture of 0·85 % (w/v) sodium chloride (4·0 ml.) and 0·2 M acetate, phosphate or veronal buffer (1·0 ml.) are given in parentheses.

Ribonuclease (pH 7·0, 0·1 mg.) was isolated from beef pancreas by the method of Kunitz (1940). It was free from phosphatase, nucleotidase, lipase, proteinase, adenine and guanine deaminase and deoxyribonuclease activities.

Deoxyribonuclease (pH 7·0, 0·1 mg. and 0·1 mg. $MgSO_4.7H_2O$ to activate) was obtained from beef pancreas by McCarty's (1946) method, and purified as described by Overend & Webb (1950). The preparation, dried from the frozen state, was free from adenine and guanine deaminase, ribonuclease, nucleotidase and phosphatase activities. Complete inhibition of the contaminating weak proteolytic (trypsin) activity was obtained, without loss of deoxyribonuclease activity, by the addition of either 0·1 M cysteine hydrochloride or 0·1 M hydroxylamine hydrochloride in 0·1 M sodium acetate (1·0 ml.) to the enzyme solution (Jacobson & Webb, 1951; Gilbert, Overend & Webb, 1951).

Intestinal phosphatase (pH 8·0, 0·5 mg.) from the mucosa of calf small-intestine was supplied by Glaxo Laboratories Ltd.

Prostate phosphatase (pH 5·0, 0·5 mg.) was isolated from surgically enucleated human prostate glands according to the method of Kutscher & Wolbergs (1935).

Pepsin (pH 2·0, 1·0 mg.) and *trypsin* (pH 8·0, 1·0 mg.) were commercial preparations (British Drug Houses Ltd.). The latter enzyme contained a weak ribonuclease activity.

Ricinus (*castor bean*) *lipase* (pH 5·0, 1·0 mg.) was prepared by the method described by Sumner & Somers (1943). It was free from nuclease and proteinase activities.

Lysozyme (pH 6·0, 0·1 mg.), isolated from egg-white as described by Alderton & Fevold (1946), was crystallized according to a method communicated by Mr E. L. French.

Hyaluronidase (pH 4·8, 0·5 mg.) was obtained by the method of Hahn (1943) and dried from the frozen state.

Cellulase (pH 7·0). Attempts to isolate this enzyme from strains of *Cytophaga* (cf. Fahraeus, 1947) were unsuccessful. An active preparation was obtained, however, from cultures of an unidentified mould. The latter was isolated by exposing cellulose pulp moistened with 0·2 M phosphate buffer (pH 7·0) to the air. Cultures were grown on filter-paper pulp for 5 days at 24°, and the cellulose and mycelium then extracted for 20 min. with 0·2 M borate buffer (pH 9·0). The suspension was centrifuged and the clear supernatant adjusted to pH 7·0. This solution, which, when activated by Mg^{++} (0·1 mg. $MgSO_4.7H_2O$ /5 ml.), hydrolysed cellulose with the formation of free-reducing groups, was used in the subsequent experiments without further purification.

Preparation of the cells

Filaments of *Cl. welchii*, *Cl. tertium*, *Cl. septicum*, *Bacillus mycoides*, *B. vulgatus* and *B. subtilis* var. *viscosus* were obtained when the organisms were separately cultivated in a magnesium-deficient Evans's peptone (2 % w/v) + glucose (0·2 % w/v) + water medium (Webb, 1948*a*) at 37°. Filamentous forms of *Lactobacillus casei* and *Lb. delbrueckii* were produced by the growth of the lactobacilli in the magnesium-deficient chemically-defined medium previously described (Webb, 1951). Cell populations composed mainly of chains were obtained when the magnesium concentration in the above media was increased to 4–6 p.p.m.

The cultures (*c.* 30 ml.) of the filaments or chains were centrifuged after 16–18 hr. incubation at 37°, and the cells then washed three times with 0·85 % (w/v) sodium chloride. Suspensions of cells in distilled water were divided into two fractions, one of which was killed at 80° by the 'flash sterilization' method of Dubos & MacLeod (1938). The second fraction was used for short-term experiments without inactivation.

The chains of *Cl. welchii, Cl. tertium* and *B. subtilis* var. *viscosus* when stained by the appropriate methods were found to be surrounded by large clearly defined capsules. However, it was apparent that the individual cells in the chains were not held together by the capsular material, since it was possible to remove the latter with 5 % (w/v) aqueous sodium cholate at 60° without affecting the morphology of the chains.

The action of hydrolytic enzymes on the cells

A measured amount of a given cell preparation was added to the enzyme solution such that the final suspension gave a turbidity reading of approximately 0·8 (=*c*.150–200 μg. dry weight/ml.) on the scale of the Spekker photo-electric absorptiometer. Changes in the morphology of the cells were determined from stained smears prepared initially and after 1, 2, 4, 18 and 48 hr. at 37°. Toluene (0·1 ml.) was added to the suspensions as a preservative after 4 hr.

RESULTS

Deoxyribonuclease, prostatic and intestinal phosphatases, lipase, pepsin, hyaluronidase and cellulase were without observable effect upon the morphology of either filaments or chains of the bacilli, clostridia and lactobacilli. These cells became thinner and Gram-negative on incubation with ribonuclease, owing to the loss of the ribonucleic acid of the Gram complex, but they retained their initial form. A short (i.e. up to 4 hr.) exposure of filaments or chains to the action of trypsin resulted in no observable change in morphology. Over longer periods of time (18–48 hr.), the cells became Gram-negative as a result of the action of the ribonuclease contaminant in the trypsin preparation and were then digested by the proteolytic enzyme (cf. Jones, Stacey & Webb, 1949). Thus, in these experiments, a marked decrease in the opacity of the cell suspension was observed after 24 hr., whilst the examination of stained smears revealed the presence of much amorphous Gram-negative debris.

The clostridia and lactobacilli were relatively resistant to the lytic action of lysozyme in as much as no significant clearing of the cell suspensions occurred in 24 hr. at 37°. However, the chains of these organisms were rapidly broken by lysozyme since stained smears, prepared from the cell suspensions after 2 hr. contact with the enzyme, showed isolated normal rods with only occasional chains (Pl. 1, figs. 1, 2). In contrast, lysozyme had no effect upon the appearance of the filamentous clostridia and lactobacilli even after prolonged (48 hr.) incubation although, at the end of this period, the cells were Gram-negative (cf. Webb, 1948*b*). *B. subtilis* var. *viscosus* cells after 2 hr. contact with lysozyme were considerably swollen and had a poor affinity for the Gram counterstain (carbol fuchsin). At the end of this period, however, the chain forms of the organism were broken with the formation of single, isolated rods (Pl. 1, figs. 3, 4). The appearance of the filamentous cells was unaffected by the enzyme. Similar results were obtained with the suspensions of *B. mycoides*, although, in this case, marked lysis of the cells occurred (Pl. 1, figs. 5, 6). Both the filamentous and chain forms of *B. vulgatus* were lysed completely by lysozyme.

The action of an intracellular enzyme preparation from Clostridium welchii *on the chains and filamentous forms of* Clostridium welchii

The cells from 300 ml. of a 15 hr. culture of *Cl. welchii* in 2 % (w/v) Evans's peptone water containing 0·2 % (w/v) glucose, was collected (centrifuge) and washed three times with 0·85 % (w/v) sodium chloride. A thick suspension of the cells was then shaken with glass beads in a Mickle tissue disintegrator (Mickle, 1948). When it was apparent from stained smears that the cells were 90–95 % disintegrated, the suspension was diluted with 0·85 % (w/v) sodium chloride (50 ml.) and centrifuged for 1 hr. at 6000 r.p.m. The clear supernatant was decanted and buffered at pH 7·0.

Samples of this cell-free preparation were added to suspensions of both the chain and filamentous forms of *Cl. welchii* at 37°. Stained smears were prepared from each suspension after 8 hr. Although the presence of the enzymes of the autolytic system (cf. Jones *et al.* 1949) in the extract from the mechanically disintegrated cells caused some lysis of the cell suspensions, it was seen from the smears that the extract also contained an enzyme with some properties similar to those of lysozyme. Thus, after 8 hr. incubation with the extract, only single isolated rods were observed in the smears prepared from the suspensions which initially contained chains. The filaments, however, were lysed slowly by the intracellular enzyme preparation, and fragmentation of these cells was not detected.

DISCUSSION

The fact that the action of lysozyme on the chain-forms of Gram-positive lactobacilli, bacilli and clostridia results in the liberation of the constituent cells, suggests that the latter are held together in the chains by a mucopolysaccharide-like material. This material is not the abundant capsular substance formed by the cells when grown in magnesium-deficient media, since the capsular polysaccharide may be removed by extraction with 5 % (w/v) sodium cholate solution without affecting the structure of the chains.

The separation of isolated normal rods as a result of the action of lysozyme on the bacterial chains indicates that the latter are composed of cells which have completed division in the sense of the splitting of the transverse cell walls (Robinow, 1945), but which have failed to separate. It is known that lysozyme hydrolyses a polysaccharide constituent in the cell walls of susceptible bacteria (Meyer, 1946; Welshimer & Robinow, 1949). Thus, it appears that the cell-wall material between individual cells in a given chain is in tension and breaks after a relatively short exposure to lysozyme. Alternatively, it is possible that the cell wall is destroyed completely by the action of lysozyme, and that the single swollen 'cells' observed after the action of the enzyme on the chains are isolated protoplasts. For example, Welshimer & Robinow (1949) concluded from a study of the lysis of *B. megatherium* by lysozyme that the cell wall is destroyed very early in the lytic process. The results of Welshimer & Robinow

(1949, e.g. their figs. 9 and 11) show that the chain forms of *B. megatherium* are broken by the action of lysozyme. In this case it is considered that the chains fall apart as a result of lysis of the cell wall, and the protoplasts thus liberated assume a random distribution (H. J. Welshimer, personal communication). In support of either of these hypotheses it was observed that the action of lysozyme on the (normal) chains of *Streptococcus pyogenes* (Pl. 1, fig. 7) under the conditions described in the experimental section, resulted in the separation of the constituent cells (Pl. 1, fig. 8).

The failure of the filamentous forms of the Gram-positive bacilli to undergo fission when exposed to lysozyme is in accordance with the fact that these cells in general appear as long elements which only infrequently contain transverse cell walls (Webb, 1949).

The presence of an enzyme, in extracts from mechanically disintegrated *Cl. welchii* cells, which resembles lysozyme in its action on the chain forms of *Cl. welchii* is of considerable interest. It remains to be established whether such an enzyme exists in other bacteria and plays any part in the processes of cell division. The fact that enzymes of the lysozyme type have been isolated from such widely different organisms as sarcinae (Meyer, Palmer, Thompson & Khorazo, 1936) and actinomyces (Krassilnikov & Koreniako, 1939) may be of some significance in this connexion.

Thanks are due to Prof. M. Stacey, F.R.S., and to Dr H. B. Fell for their interest in this work, and to Mr K. Barclay, B.Sc., for the preparation of the mould cellulase. Grateful acknowledgement is made to Mr E. L. French, M.Sc., of the Walter and Eliza Hall Institute for Medical Research, Melbourne, for details of the preparation of lysozyme, and for the gift of a crystalline specimen of the enzyme. The work was in part financed by a grant from the Medical Research Council.

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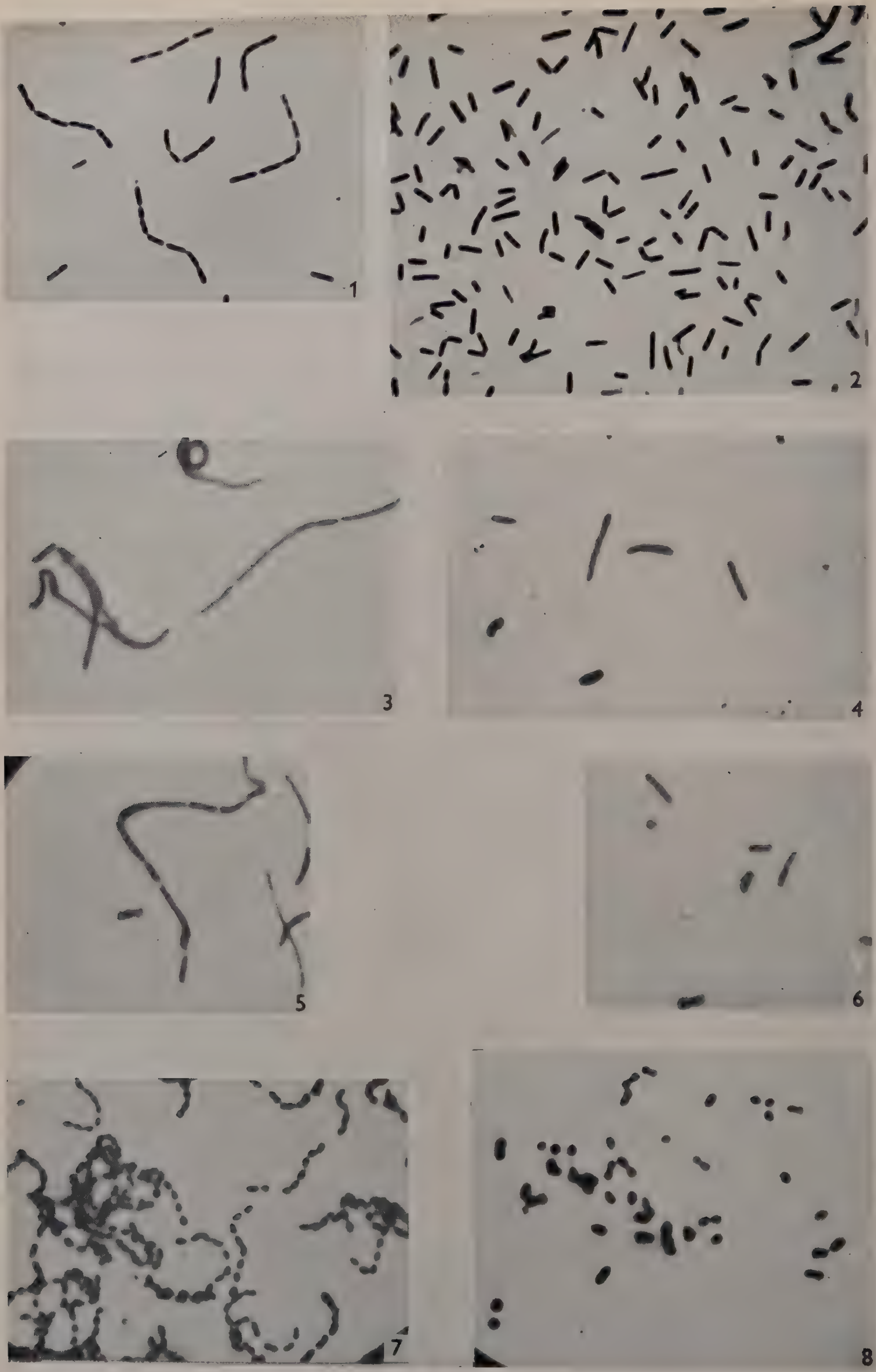

Figs. 1–8

M. Webb—Action of enzymes on bacterial morphology. Plate 1

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EXPLANATION OF PLATE

Fig. 1. Chain forms of *Cl. welchii* harvested from an 18 hr. culture in an Evans's peptone water medium deficient in magnesium (×1000).

Fig. 2. *Cl. welchii* cells as shown in fig. 1, after 2 hr. in the presence of lysozyme (×1000).

Figs. 3, 4. Chain forms of *B. subtilis* var. *viscosus* before (fig. 3) and after (fig. 4) treatment with lysozyme for 2 hr. at 37° (×1400).

Figs. 5, 6. Chain forms of *B. mycoides* before (fig. 5) and after (fig. 6) treatment with lysozyme for 2 hr. at 37° (×1400).

Fig. 7. *Strep. pyogenes* cells from a 24 hr. culture in 2 % (w/v) Evans's peptone water (×1400).

Fig. 8. *Strep. pyogenes* after treatment with lysozyme for 2 hr. at 37° (×1400).

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Growth Requirements of *Schizosaccharomyces octosporus*, a Yeast Exacting towards Adenine

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SUMMARY: The growth requirements of a strain of *Schizosaccharomyces octosporus* were studied by auxanographic and tube tests. The organism grows satisfactorily on a glucose + mineral salts medium containing asparagine and ammonium sulphate, provided also that inositol, pantothenic acid, nicotinic acid, biotin, histidine, methionine and adenine are added. Complex interactions between adenine and guanine were found, and for this reason *S. octosporus* cannot be regarded as a suitable test organism for the assay of adenine.

The present investigation arose from studies still in progress of the inositol requirements of a number of yeasts. Twelve strains were examined, and growth of eleven of them was satisfactory when inositol or yeast extract was added to a defined medium containing glucose + mineral salts, ammonium sulphate, casein hydrolysate and B vitamins. The exception was *S. octosporus* which failed to grow in absence or presence of inositol. Further investigations showed that *S. octosporus* required adenine, not hitherto reported as an essential metabolite for yeasts, and its growth requirements were therefore studied in more detail.

METHODS

Tube tests. The usual procedure for microbiological assay was followed, but in place of bacteriological tubes, flat-bottomed tubes (Samco), 4 × $\frac{3}{4}$ in., were used. Tubes were selected so that the internal diameter was within the limits 1·66 and 1·72 cm. The tubes, containing 6 ml. of test medium and covered with loosely fitting glass caps, were sterilized for 10 min. at 10 lb. pressure. After inoculation, they were incubated in a vertical position at 25° in a water-bath.

Estimation of amount of yeast growth. The tubes were fitted into a black wooden container open at opposite sides which held them in the optical beam of the Spekker absorptiometer, in the place normally occupied by the glass cells. Thus turbidity readings could be taken from the same tube at intervals during the growth of the organism, without exposing the contents to contamination.

Test organism and inoculum. The test organism, carried on malt agar slopes, was subcultured weekly or more frequently as required. In the preparation of the inoculum a loopful of a 24 hr. culture was suspended in 6 ml. of basal medium to give a turbidity in the tubes reading between 0·15 and 0·25 in the Spekker (i.e. approximately 0·2–0·4 mg. dry weight yeast/6 ml.). For inoculation one drop (20 μl.) of the suspension was added to each tube from a sterile Pasteur pipette.

Auxanographic tests. See Pontecorvo (1949); Cutts & Rainbow (1949).

Media. Basal medium I. Composition per 500 ml. final volume was, in g.:

glucose, 10·0; L-asparagine, 1·0; KH_2PO_4, 0·75; $CaCl_2.6H_2O$, 0·25; $MgSO_4.7H_2O$, 0·25; $(NH_4)_2SO_4$, 1·0; in μg.: aneurin HCl, 250; pyridoxin HCl, 250; Ca D-pantothenate, 250; nicotinic acid, 250; D-biotin, 0·1; KI, 50; in ml.: trace element solution (Emery, McCleod & Robinson, 1946), 0·5; lactate buffer, 5·0. The lactate buffer was prepared by adjusting 2·5 ml. lactic acid (syrupy A.R.) to pH 5·0 with KOH and making up to 5 ml.

Basal medium II. As for medium I with the addition of: inositol, 3·33 mg.; DL-methionine 8·33 mg.; L-histidine HCl, 4·17 mg./500 ml. of medium.

Basal medium III. As for medium I, with the addition of 1 g. casein hydrolysate/500 ml. of medium, the weight being based on the amino-N content (×6·25) of a solution prepared from Glaxo vitamin-free casein (Barton-Wright, 1946).

RESULTS

Tube tests with twelve yeasts. In these experiments basal medium I was used. Series of tubes were set up containing basal medium only, basal medium + 6·7 μg. inositol/ml., and basal medium + 1 mg. Difco Bacto yeast extract/ml. After sterilization and cooling, the various sets were inoculated, and duplicate tubes read in the Spekker at intervals of 22, 30, 47, 72 and 120 hr. Table 1 summarizes the results obtained at 72 hr.

Table 1. *The effect of inositol and yeast extract in basal medium I on the growth of twelve yeasts*

	Basal medium I with		
	No addition	Inositol 6·7 μg./ml.	Yeast extract 1 mg./ml.
Organism	Spekker readings at 72 hr.		
Kloeckera brevis	0	1·21	1·37
K. apiculata	0	1·19	1·40
Torula utilis major	1·18	1·23	1·28
Saccharomyces carlsbergensis	1·40	1·51	1·58
S. intermedius	1·00	1·39	1·58
S. exiguus	1·49	1·49	1·65
S. validus	0·09	0·74	1·55
S. turbidans	1·36	1·43	1·63
S. pastorianus	0·43	1·40	1·63
Bottom yeast	0·23	0·87	1·00
Schizosaccharomyces pombe	0	1·25	1·40
Schizosacc. octosporus	0	0	0·90

Of all the yeasts studied only *S. octosporus* failed to grow in the basal medium alone and in the presence of inositol. The extent of growth in the basal medium + yeast extract plainly indicated the presence in the latter of substances essential for this yeast, and which were lacking in the basal medium. By comparison with more complex liquid media employed in microbiological assays, for example, the deficiencies appeared likely to be amino-acids or purine-pyrimidine bases.

Auxanographic tests with S. octosporus. In a preliminary survey the following substances in various combinations were added to auxanographic plates:

casein hydrolysate, inositol, *p*-aminobenzoic acid, riboflavin, folic acid, adenine, guanine and uracil. It was clear from the results of these experiments that growth took place only when casein hydrolysate, inositol and adenine were added together. Accordingly, the requirements for adenine and amino-acids were examined.

Effect of purine-pyrimidine bases. Series of tubes were prepared with basal medium III (6 ml.) + 6·7 μg. inositol/ml. of medium. Sets were also made up to contain in addition to the foregoing, in μg./ml. of medium: (i) 4·2, 8·3, 16·7 adenine; (ii) 8·3 each of adenine, guanine and uracil; (iii) 8·3 each of guanine and uracil; (iv) 1000 Difco Bacto yeast extract. After sterilization, the tubes were inoculated with a 24 hr. inoculum of *S. octosporus* and the turbidities measured at intervals, with the results shown in Table 2.

Table 2. *The effect of purine-pyrimidine bases in basal medium III on the growth of* Schizosaccharomyces octosporus

(Supplements indicated as follows: I = inositol; A = adenine; G = guanine; U = uracil; YE = Difco Bacto yeast extract. Amounts in μg./ml. medium indicated by numbers.)

	Period of incubation, hrs.				
	48	55	74	96	142
Basal medium III plus	Spekker readings				
6·7 I	0	0	0	0	0
6·7 I + 4·2 A	0·31	0·47	0·64	0·74	0·85
6·7 I + 8·3 A	0·34	0·58	0·87	1·01	1·12
6·7 I + 16·7 A	0·35	0·60	1·10	1·24	1·29
6·7 I + 8·3 each of A, G, U	0·34	0·55	1·00	1·19	1·27
6·7 I + 8·3 each of G, U	0	0	0·02	0·05	0·53
6·7 I + 1000 YE	0·63	0·80	1·02	1·13	1·17

The marked stimulation of growth due to adenine was clearly shown, there being no growth in the absence of purines and pyrimidines. With guanine and uracil together growth proceeded fairly rapidly after a 96 hr. lag, while with 8·3 μg. each of adenine, guanine and uracil growth equivalent approximately to 16·7 μg. adenine was obtained. Yeast extract increased the rate of growth in the early stages; but it seemed to contain insufficient purines and pyrimidines for optimal growth of the organism.

Effects of purine and pyrimidine bases in admixture. These experiments suggested the investigation of the separate and combined effects of the purines adenine, guanine and xanthine, and the pyrimidines uracil, thymine and cytosine. Casein hydrolysate could be satisfactorily replaced by methionine and histidine (see below), and accordingly basal medium II was used in the experiment. Purines and pyrimidines in various combinations were added at the rate of 16·7 μg./ml. of medium to sets of tubes which respectively contained: (i) no added adenine; (ii) 0·83 μg. adenine/ml.; (iii) 33·3 μg. adenine/ml. of medium. The following effects were observed: (*a*) good growth took place after 96 hr. in presence of guanine and absence of adenine; (*b*) guanine (16·7 μg./ml.) exerted a strong inhibitory effect on growth in presence of 0·83 μg. adenine/ml., particularly during the first 96 hr.; (*c*) xanthine in presence of 0·83 µg. adenine/ml. had a stimulatory effect on growth.

Further investigation of the interrelations of adenine and guanine revealed that the effects observed were dependent on the quantitative relationships of the two bases. At suboptimal levels of adenine, the effect of increasing amounts of guanine was a stimulation which reached a maximum when the molar ratio of guanine to adenine was about 2:1. At this ratio, the effect of guanine was equivalent to that of a similar quantity of adenine. For example, 2 µg. guanine + 1 µg. adenine produced a response equal to that of 3 µg. adenine. When the ratio of guanine to adenine increased beyond 2:1 inhibition occurred, and at a molar ratio of 10:1 inhibition was almost complete. These results are shown in Fig. 1.

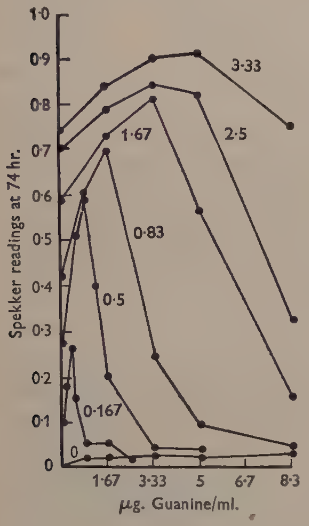

Fig. 1

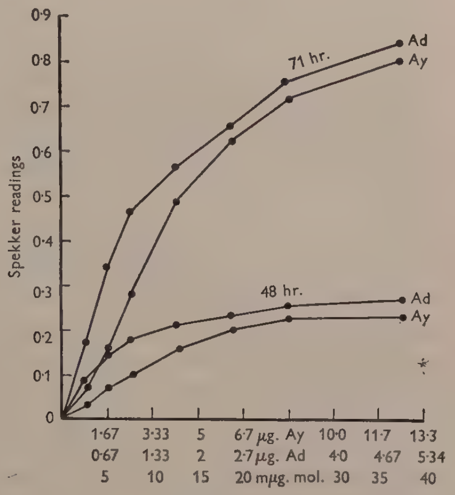

Fig. 2

Fig. 1. Interaction of adenine and guanine. Figures on curves indicate amounts of added adenine µg./ml.

Fig. 2. Comparison of effects of adenine and adenylic acid. Ad = with adenine, Ay = with adenylic acid. Ratio of molecular weights of adenylic acid and adenine is approximately 2·5.

With mixtures of xanthine and adenine, no inhibitory effect due to xanthine was revealed, and relatively large amounts of xanthine were required for stimulation. Thus 150 µg. xanthine when added to 5 µg. adenine produced growth equivalent to that obtained with about 10 µg. adenine.

Effect of adenylic acid. Adenylic acid was found to replace adenine, but it was less effective on a molar basis, particularly at the lower dosage levels (Fig. 2).

Amino-acid requirements of S. octosporus. The amino-acid requirements of the organism were determined qualitatively by auxanographic tests which indicated clearly the essential nature of methionine and histidine. Tube-test experiments were then carried out which showed that in absence of both amino-acids no visible growth took place within 90 hr., and was only just observable at 140 hr. With 33·3 μg. methionine/ml., growth commenced at 70 hr., and with the same amount of histidine growth was observed at 50 hr., and reached a slightly higher limit than that attained with 2 mg. casein hydrolysate/ml. (Fig. 3). The apparent lack of adequate amounts of histidine and methionine in the casein hydrolysate could not be compensated by increasing the amount of casein hydrolysate in the medium. On the contrary, increasing amounts of casein hydrolysate involved a certain degree of inhibition of growth. This may be due to the presence of increasing amounts of arginine and lysine which were observed to have a marked inhibitory effect both in auxanographic and tube experiments.

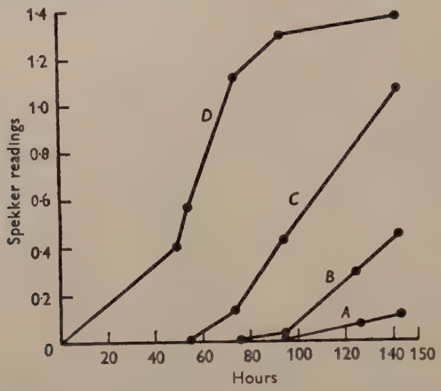

Fig. 3. Effect of methionine and histidine. *A*, without amino-acids; *B*, with methionine; *C*, with histidine; *D*, with methionine and histidine. Each amino-acid added at level of 33·3 μg./ml.

Vitamin requirements of S. octosporus. Tube tests were used to examine the vitamin requirements, with a basal medium III containing 16·7 μg. adenine/ml., and all the vitamins B except that which was being studied. The organism required inositol, pantothenic acid, nicotinic acid and biotin; pyridoxin, *p*-aminobenzoic acid, riboflavin and folic acid did not appear to be necessary, and aneurin had a slight stimulatory effect. The addition of 1 mg. Difco Bacto yeast extract/ml. to basal medium III containing 16·7 μg. adenine/ml. and 6·7 μg. inositol/ml. produced only a very slight increase in growth, suggesting that this medium is essentially complete.

DISCUSSION

The fact that the strain of *S. octosporus* examined can grow normally only in the presence of adenine suggests that this organism is deficient in its ability to synthesize adenine from simpler constituents. Guanine is also required, and if not already present in the medium may be readily produced by conversion of the equivalent amount of adenine. Support for this suggestion is derived from the following observations: (*a*) in the presence of guanine alone the organism can grow only after a lag phase of some 4 days, presumably as a result of a slow conversion of guanine to adenine; (*b*) but when guanine is added to a suboptimal concentration of adenine growth is improved to an extent similar to that which would have been obtained by addition of an equivalent amount of adenine, provided that the molar ratio of guanine to adenine does not exceed 2:1.

In the study of a *Neurospora* mutant requiring adenine, Fairley & Loring (1949) concluded that guanine was synthesized from adenine, and pointed out that the biosynthesis of guanine is analogous to that reported by Brown, Roll, Plentl & Cavalieri (1948) for the white rat. In this case, experiments with labelled adenine and guanine showed that whereas adenine could be converted to guanine, the reverse change was not effected. Our results with *S. octosporus* show that this strain is able to achieve the conversion in both directions, and apparently differs in this respect from the *Neurospora* of Fairley & Loring. Although these workers produced no evidence that their organism could utilize guanine, this might have been forthcoming had growth been studied over a longer period.

It has been shown that maximum stimulation and inhibition by guanine depends on the ratio guanine : adenine over a wide range of concentrations and not on the absolute values. It seems possible, therefore, that they, or their metabolic products, are competing for an enzyme system.

A strain of *S. cerevisiae*, designated 'Yeast 47', reported by Cutts & Rainbow (1949), can grow in the absence of *p*-aminobenzoic acid when adenine, methionine and histidine are supplied. They suggested that *p*-aminobenzoic acid may form part of a co-enzyme involved in the synthesis of these substances. While *S. octosporus* is not exacting towards *p*-aminobenzoic acid (and there is evidence that it can synthesize it), it still requires preformed adenine, methionine and histidine. This suggests that there is a further stage between *p*-aminobenzoic acid and adenine, methionine and histidine which 'Yeast 47' can, but *S. octosporus* cannot, negotiate.

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The Kinetics of Pyruvate Production by *Aerobacter aerogenes*

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SUMMARY: Pyruvic acid accumulates in aerated cultures of *Aerobacter aerogenes* during logarithmic growth and disappears rapidly in the stationary phase. The amount accumulated depends upon the carbon source utilized, being greatest with glucose and malic acid and least with acetic acid, of the compounds studied. For aerated washed suspensions of cells trained to metabolize glucose, malate, fumarate or succinate respectively, the rates of pyruvate production for various substrates are in the order: rate in malate > fumarate > succinate > acetate. Only cells trained to metabolize acetate produce pyruvate from acetate as fast as from succinate and fumarate. When ammonium salt solution is added to these suspensions accumulation of pyruvate ceases.

Rates of pyruvate production at various substrate concentrations by washed suspensions accord with a kinetic scheme in which pyruvate appears as a transitory intermediate in a reaction sequence. Results are not compatible with the existence of the tricarboxylic acid cycle in bacteria.

The operation of the tricarboxylic acid cycle in bacterial respiration has not been proved (Ajl & Werkman, 1949), although a number of investigations carried out with *Escherichia coli* and *Aerobacter aerogenes* grown on simple chemically-defined media suggest that it may play a part in the cell economy. Ajl & Werkman (1948, 1949) showed that a number of compounds known to participate in the cycle partially abolished the carbon dioxide requirement of cultures grown both aerobically and anaerobically. Dagley, Dawes & Morrison (1949, 1950*a*) showed that small additions of these compounds or of their related amino-acids accelerated the growth of *A. aerogenes* in a glucose ammonium salt medium having access to normal atmospheric CO_2, either by decreasing lag periods or by increasing initial growth rates. The latter observations were, however, not regarded as evidence for the existence of the full cycle in cultures of *A. aerogenes*, since some of the participating compounds retarded growth, and fluoroacetate, which might be expected to stop growth by blocking the cycle, had in fact little effect. The importance of particular members of the cycle (pyruvic, oxaloacetic and α-ketoglutaric acids) was emphasized by the identification of alanine, aspartic and glutamic acids in the metabolizing fluids after growth in glucose ammonium salt medium (Dagley, Dawes & Morrison, 1950*b*). Baskett & Hinshelwood (1950) grew *A. aerogenes* in chemically-defined media with members of the cycle as sole sources of carbon and, from observations on the degree of training necessary to provide maximum rate of growth on each compound, and from the cross-training also achieved, concluded that the cycle represented a highly significant pattern among the carbon compounds supporting growth.

Extensive investigations by Wood, Werkman and their collaborators during the past decade have elucidated many relationships between pyruvic, oxaloacetic, malic, fumaric, succinic and acetic acids in bacterial metabolism. However, this significance is not confined to the problem of assessing the part played by the tricarboxylic acid cycle in these systems. As Barron (1943) pointed out, pyruvic acid is the hub from which radiates a great variety of metabolic activities; and training to metabolize various carbon sources may, for example, be interpreted by reference to the ease with which cultures achieve a production of pyruvate adequate for growth requirements. In the present work we have related the growth of *A. aerogenes* on various single carbon sources with the rate at which it produces pyruvic acid from them and with its ability to oxidize these substrates completely.

EXPERIMENTAL

The organism used throughout this work was *A. aerogenes* N.C.T.C. 418. Media were prepared from the following stock solutions: solution A, 9 g. KH_2PO_4, 2 g. $(NH_4)_2SO_4$/l. glass-distilled water, and adjusted to pH 7·1; solution B, 9 g. KH_2PO_4/l., pH 7·1; 1 % (w/v) solutions of carbon compounds (glucose or organic acids), the pH of the latter being adjusted to 7·1 with NaOH; 10 % (w/v) solution of $MgSO_4.7H_2O$. Solutions were sterilized separately and mixed on cooling. Media for training were made up in Pyrex boiling tubes (6 × 1 in.) from 15 ml. solution A, 10 ml. solution of carbon source and 0·1 ml. $MgSO_4$ solution, incubated at 37° and aerated with a stream of sterile air. At least ten serial subcultures were given in training a particular strain to metabolize a given carbon compound. Methods of obtaining growth curves and precautions necessary to ensure reproducibility have already been described (Dagley, Dawes & Morrison, 1950*a*, *c*). Washed bacterial suspensions were prepared by harvesting after growth in the defined media and washing three times with solution B. Certain experiments with washed suspensions were carried out in large Pyrex tubes (8 × 1½ in.).

Pyruvic acid was determined by the toluene extraction method of Friedemann & Haugen (1943). A calibration curve was prepared with freshly distilled pyruvic acid using a Spekker photoelectric absorptiometer with Ilford filters (neutral H 508 and green OG 1).

Oxygen uptakes were measured with the conventional Warburg apparatus.

RESULTS

Production of pyruvate during growth

Media containing 15 ml. solution A, 0·1 ml. 10 % (w/v) $MgSO_4.7H_2O$ and 10 ml. of a 1 % (w/v) solution of the appropriate carbon source were inoculated with 0·1 ml. of a culture which had grown, after training, in an aerated defined medium containing the same source of carbon. The sources of carbon were the following organic acids: malic, lactic, citric, fumaric, succinic and acetic. The pyruvic acid concentrations of the culture fluids were estimated during growth on 5 ml. samples of culture withdrawn at intervals and centrifuged rapidly. The results are shown in Fig. 1, where pyruvate concentrations are plotted against the logarithms of the corresponding bacterial populations in order to effect a comparison between cultures growing at different rates and to different populations. Growth curves showing sharply defined growth and stationary phases were obtained as described previously (Dagley *et al.* 1950*a*, *c*). It is seen that the rise in pyruvate concentration during growth was succeeded by a sharp fall when the stationary phase was reached, although in the case of succinate and acetate cultures the amount of pyruvate formed at any stage was barely detectable. We have already reported similar variation in pyruvate concentration in aerated glucose ammonium salt media (Dagley *et al.* 1950*b*).

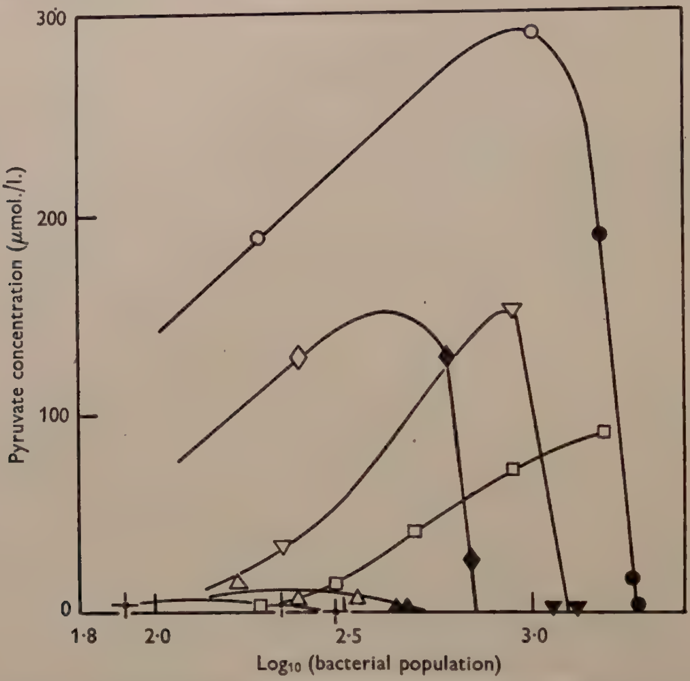

Fig. 1. Pyruvate production during growth on various organic acids: malic, ○; lactic, ◇; citric, ▽; fumaric, □; succinic, △; acetic, -+-. Blacked-in symbols indicate values in stationary phase.

Production of pyruvate by washed bacterial suspensions from various substrates

If pyruvate is an essential intermediate in processes regulating cell division its accumulation during the logarithmic phase may be due to a rate of production in excess of the requirements of the cells, and its disappearance in the stationary phase to exhaustion of the substrate from which it arose. To demonstrate definite pyruvate production from acetate and succinate the ammonium salt was omitted from the medium, and washed cells were used so that pyruvate might accumulate, utilization in cell division being precluded. Under these conditions it was immediately possible to demonstrate the production of pyruvate from acetate and succinate. The investigation was then extended as follows. Washed bacterial suspensions were prepared of cells trained to metabolize glucose, succinate, fumarate, malate or acetate, and each batch of cells was then tested for its ability to produce pyruvate. Five sets of large tubes were set up, each containing 40 ml. solution B, 0·4 ml. 10 % (w/v) $MgSO_4.7H_2O$ solution; equimolar solutions of the various carbon sources were used, 40 ml. per tube being added to the respective sets. When the 20 ml. of cell suspension to be tested had been added, the concentration of substrate was 0·028 M in every case. Each tube was then aerated at 37° by a stream of sterile air at five bubbles per second, and at suitable intervals 5 ml. samples were withdrawn, rapidly centrifuged and the pyruvate concentrations of the supernatants determined. Curves for the development of pyruvate concentration with time were obtained for each suspension; those for cells trained to succinate are given in Fig. 2. From such curves the rates of increase of pyruvate concentration were obtained, and in Table 1 are expressed in each case relative to the rate in acetate.

Table 1. *Relative rates of pyruvate production by cells trained to metabolize various sources of carbon*

(Figures in brackets give actual pyruvate production from acetate, in μmol./l./hr., for the different batches of cells.)

	Substrates against which cells were tested			
	Malate	Fumarate	Succinate	Acetate
Substrates to which cells were trained	Relative rates of pyruvate production			
Glucose	20	4	3	1 (7)
Malate	23	13	5	1 (11)
Fumarate	15	12	9	1 (15)
Succinate	45	24	9	1 (7)
Acetate	5	1	1	1 (80)

It is seen that, in general, the pyruvate production from acetate was slower than from any other substrate. Only in the case of those cells which had been trained to acetate was the rate of production comparable with the rest.

Towards the end of each experiment the medium was completed (for growth) by the addition of 1·2 g. $(NH_4)_2SO_4$/l. In every case this addition resulted either in a fall of pyruvate concentration or in the arrest of the steady rise which was occurring prior to the addition. When there was a definite fall in pyruvate concentration, turbidity measurements indicated that cell division had begun.

Inocula of 0·1 ml. of suspensions of washed cells trained to the various substrates were tested for their ability to grow in 25 ml. lots of media, each containing respectively one substrate as sole carbon source. We confirmed the observations of Baskett & Hinshelwood (1950) that only cells trained to acetate were able to utilize this compound readily, and found that adaptation to earlier members of the series (acetate, succinate and fumarate) resulted in adaptation to a later member but not to an earlier one.

Effect of different substrate concentrations

In the foregoing experiments with washed cells, the rates of pyruvate production were approximately linear. In order to ascertain whether a maximum pyruvate concentration might eventually be reached, thicker suspensions of higher activities were used and substrate concentrations were varied. Curves for the production of pyruvate from succinate at various concentrations by cells trained to succinate are given in Fig. 3. Curves of similar form have also been obtained for other substrates.

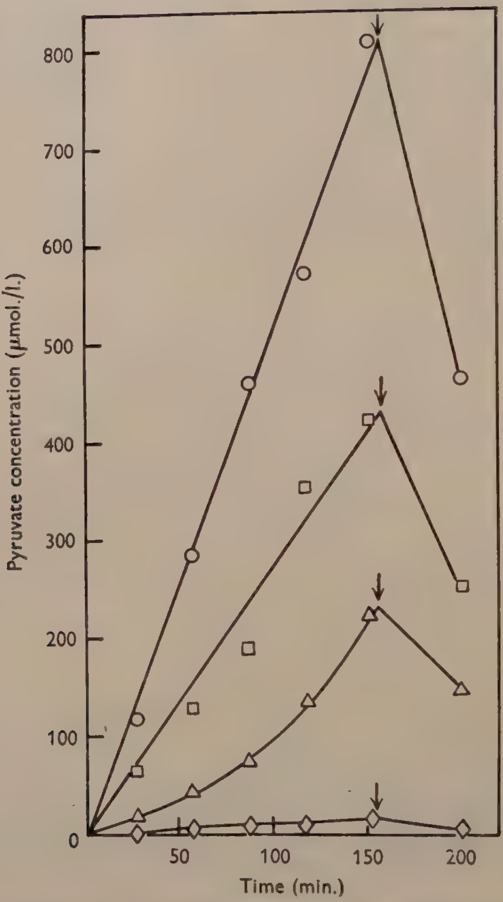

Fig. 2. Pyruvate production by non-proliferating suspensions from various organic acids: malic, ○; fumaric, □; succinic, △; acetic, ◇. Arrows indicate addition of 1·2 g. $(NH_4)_2SO_4$/l.

Although the pyruvate concentration reaches a higher maximum the higher the concentration of substrate, no stoichiometrical relationship exists. Thus in Fig. 3, at initial succinate concentrations of 2060, 6180, 10,300 and 14,420 μmol./l., the respective maximum pyruvate concentrations are 57, 323, 630 and 940 μmol./l. When the cells are aerated in phosphate buffer with pyruvate, the latter is rapidly decomposed. For example, a concentration of 7600 μmol. pyruvate/l. was decreased to 150 μmol./l. in 50 min. These facts indicate that the accumulation of pyruvate in the medium is the result of a rate of production in excess of the rate of decomposition, a maximum being attained when the rates are equal, due to the fall in concentration of the initial substrate and the increase in concentration of the pyruvate.

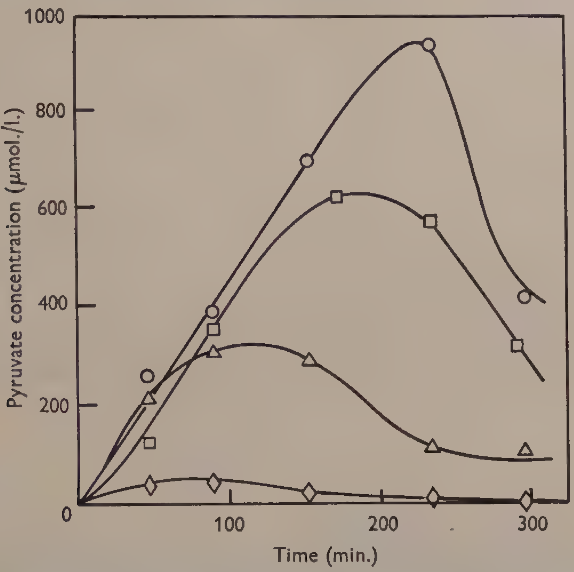

Fig. 3. Pyruvate production by non-proliferating suspensions from succinic acid at various initial concentrations (mM): 2·06, ◇; 6·18, △; 10·30, □; 14·42, ○.

Rates of oxidation and pyruvic production

Non-proliferating cell suspensions of many micro-organisms, including *A. aerogenes*, have been shown to be capable of oxidizing to completion the substrates under investigation, except for a proportion which is assimilated (see Clifton, 1946). Measurement of the O_2 uptake of cells trained to fumarate, with fumarate, succinate and acetate as equimolar (0·028 M) substrates in the Warburg apparatus, gave values of 77, 72 and 56 μl./60 min. respectively. The same cell suspension aerated with the same substrates in boiling tubes gave, respectively, the following rates of pyruvate production: 281, 236 and 15 μmol./l./60 min.

Bearing in mind that the theoretical O_2 uptakes for fumarate, succinate and acetate are in the ratio 6:7:4, it is clear that the difficulty experienced by fumarate-trained cells in producing pyruvate from acetate is not related to the rate at which they oxidize the substrate to completion.

DISCUSSION

Baskett & Hinshelwood (1950) found that cells trained to glucose could adapt with increasing difficulty, as reflected in the training they required, to the series malate, fumarate, succinate and acetate, and we have found that for a given batch of cells, except those trained to acetate, the rates of pyruvate production from these substrates by washed suspensions followed the order, malate > fumarate > succinate > acetate. Training to grow on acetate, however, also stimulated ability to produce pyruvate from acetate, and it appears that cell division is related to the ability to synthesize this key metabolite at a rate greater than its rate of decomposition. The function of pyruvate as a growth intermediate is shown by the addition of ammonia to complete the medium, when the cells immediately draw on the accumulated pyruvate for their growth requirements. Glucose and malate, which are rapidly decomposed to pyruvate by cell suspensions, evidently permit pyruvate production in excess of growth requirements in the logarithmic phase, but in experiments with cultures growing on succinate and acetate, with lower rates of production of pyruvate by washed cells, pyruvate was barely detectable in the metabolizing fluids.

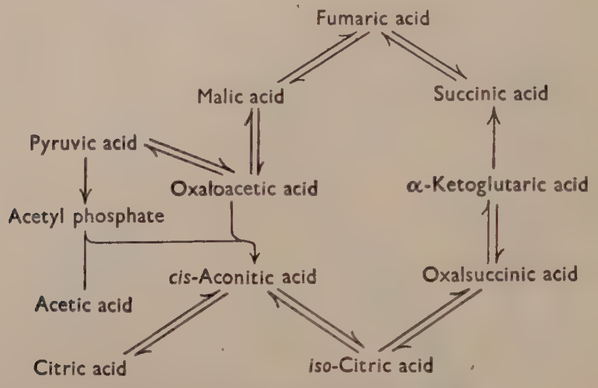

Fig. 4. The tricarboxylic acid cycle.

The complete oxidations of malate, fumarate and succinate probably proceed by way of pyruvate, and it is of general interest that the curves of Fig. 3 take the form predicted for an intermediate in a reaction-sequence and exhibit maxima dependent upon initial substrate concentration as the kinetics of such a system require (Glasstone, 1946).

If the tricarboxylic acid cycle (Fig. 4) be accepted as the mechanism for the complete oxidation of these substrates, the maxima in Fig. 3 might correspond to the complete establishment of the cycle. This mechanism, however, is not compatible with our findings, for the following reasons. Washed suspensions of acetate-trained cells give a linear production of pyruvate immediately on aeration. In phosphate buffer alone, with no added source of carbon, no pyruvate is produced and no significant amount of oxaloacetic acid (its precursor in the cycle) can, therefore, be present. An accumulation of pyruvate in the early stages requires either the production of oxaloacetate in excess of the requirements of the cycle, or the presence of appreciable amounts of oxaloacetate initially. The latter, as we have seen, is ruled out, and it is impossible for oxaloacetate to accumulate solely by the reactions summarized in Fig. 4 and, at the same time, to fulfil the demand of the cycle that one oxaloacetate molecule be consumed for each molecule formed from acetate. Furthermore, although the cycle is a respiratory mechanism, we found that fumarate-trained cells were able to oxidize succinate, fumarate or acetate at comparable rates but had great difficulty in producing pyruvate from acetate, although they did so readily from the other two substrates. These observations present no difficulty of interpretation if the substrate is oxidized via pyruvate and acetate by a non-cyclic process, and if the conversion of acetate to pyruvate takes place by an independent mechanism which only training to acetate can fully develop. Although Baskett & Hinshelwood (1950) concluded that the cycle represented a highly significant pattern among the carbon compounds used in their studies of training, they did not claim the complete operation of the cycle as the mechanism of bacterial respiration. Indeed, they concluded that 'some stretches appear to be utilized only to a small extent by cells grown in glucose', and we may conclude from our studies that the sequences resulting in the formation of pyruvic acid are of prime importance. When acetate is the substrate a mechanism such as that of the cycle, which involves prior reaction with a product of respiration, is not acceptable, and a possible alternative is that suggested by Slade & Werkman (1943) in which succinate, and hence pyruvate, can be produced by the reaction between two molecules of acetic acid.

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Blender for Preparation of Mycelial Inocula

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SUMMARY: In this blender the container and cutter assembly can be removed for heat sterilization. A cotton-wool gland maintains sterility during blending.

For certain types of mycological work undertaken in these laboratories it has been found convenient to use mycelial inocula prepared by breaking up mycelial mats, from surface cultures, in a blender. For such purposes a blender is needed which can be heat-sterilized before use and in which sterility can be maintained during use. For this purpose we have developed, and used for 3 years, a blender which we consider may have wider applications.

The commercial blenders of our experience are unsuitable. Bottom drive blenders cannot be kept clean without stripping the gland bearing. Nor can blenders with a self-lubricated bearing be repeatedly heat-sterilized because this sweats out the oil from the sintered metal. Top drive blenders do not allow for the sterilization of the shaft in the container. Moreover, in most blenders there is no provision for blending small quantities.

The container of our blender (Fig. 1) is shaped to deal effectively with quantities from 20 to 200 ml. Baffle strips are attached to the wall to decrease the spinning of the liquid. The lid screws on, and the container is held in alinement with the shaft and motor by screwing into the base of the stand. Container, lid, shaft and cutters are made of stainless steel. To allow the upper end of the shaft to be inserted into the coupling, the electric motor which runs at 6000 r.p.m. can be raised by a screw thread. The square supporting column ensures alinement of the cutters in the centre of the container. The gland is packed with cotton-wool before sterilization. The inside of the gland has a right-hand thread and this, with the clockwise rotation of the shaft, keeps the cotton-wool in position during blending. The direction of rotation also tends to tighten the right-hand thread of the shaft coupling.

The sterilized container with shaft is screwed into the base with the motor raised. The halves of the coupling are screwed together and the motor lowered.

Two minutes' blending time is usually sufficient.

There are no mechanical hazards, because the container surrounds the cutters when they are moving.

Tests of sterility during operation

The sterilized blender containing 50 ml. of sterile nutrient broth was run for 30 min. During the last 5 min. the top of the container was sprayed with a 24 hr. broth culture of *Serratia marcescens* to ensure a heavily contaminated atmosphere. No colonies appeared on any test plate after 5 days' incubation.

On no occasion have we had evidence that living mycelium is driven out of the container during operation of the blender.

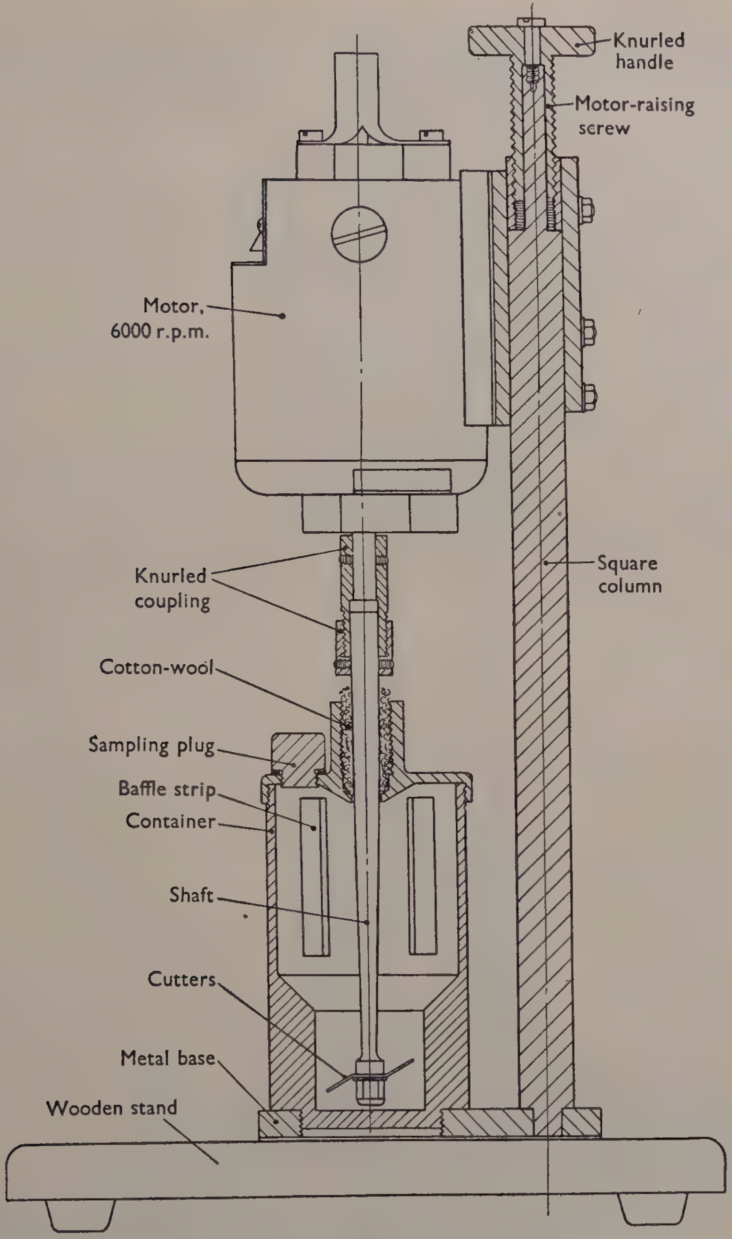

Fig. 1

Details of construction

Container. This is machined to $2\frac{3}{4}$ in. O.D. from a solid bar of Firth Vickers E.M.S. Staybrite. The four $\frac{3}{8} \times \frac{1}{8}$ in. baffle strips are each pressed on with two $\frac{1}{16}$ in. diameter dowel pins. The internal depth is $4\frac{1}{2}$ in. and the internal diameters $2\frac{1}{2}$ and $1\frac{1}{2}$ in.

Cover. Machined from the same material as the container. The total depth is $1\frac{3}{8}$ in. and the outer edge is knurled. The 1 in. diameter projection is screwed internally $\frac{3}{4}$ in. B.S.F.; the diametrical clearance of the hole on the shaft being $\frac{1}{32}$ in. A $\frac{1}{2}$ in. B.S.F. plug and rubber washer seal the sampling hole. A rubber washer may be inserted between the container and the cover but we have not found this necessary.

Shaft assembly. The tapered shaft is $\frac{3}{8}$ in. diameter at the coupling. Two cutters registering on two flats on the shaft are secured by a nut. One cutter is straight and the other bent, and the leading edges of both are sharp. The half coupling is in true alinement with the shaft and secured to it by two grub screws. All these parts are made of Staybrite.

Motor. This is a series-wound $\frac{1}{30}$ h.p., 240 V. a.c., S.P. motor, rated at 2000 r.p.m. This speed is for the motor fully loaded, and under the light loading in the blender it runs at approximately 6000 r.p.m.

Stand. A brass plate supporting a $\frac{13}{16}$ in. square column is screwed to a wooden base mounted on rubber feet. The motor bracket, incorporating a nut, is a good sliding fit on the column. The knurled handle with screw is attached to the top of the column but is free to rotate.

This blender can easily be made in a laboratory machine shop and the authors will be pleased to supply full constructional details to those interested.

We thank Miss Anne Levy for her most useful criticisms and for the sterility tests described.

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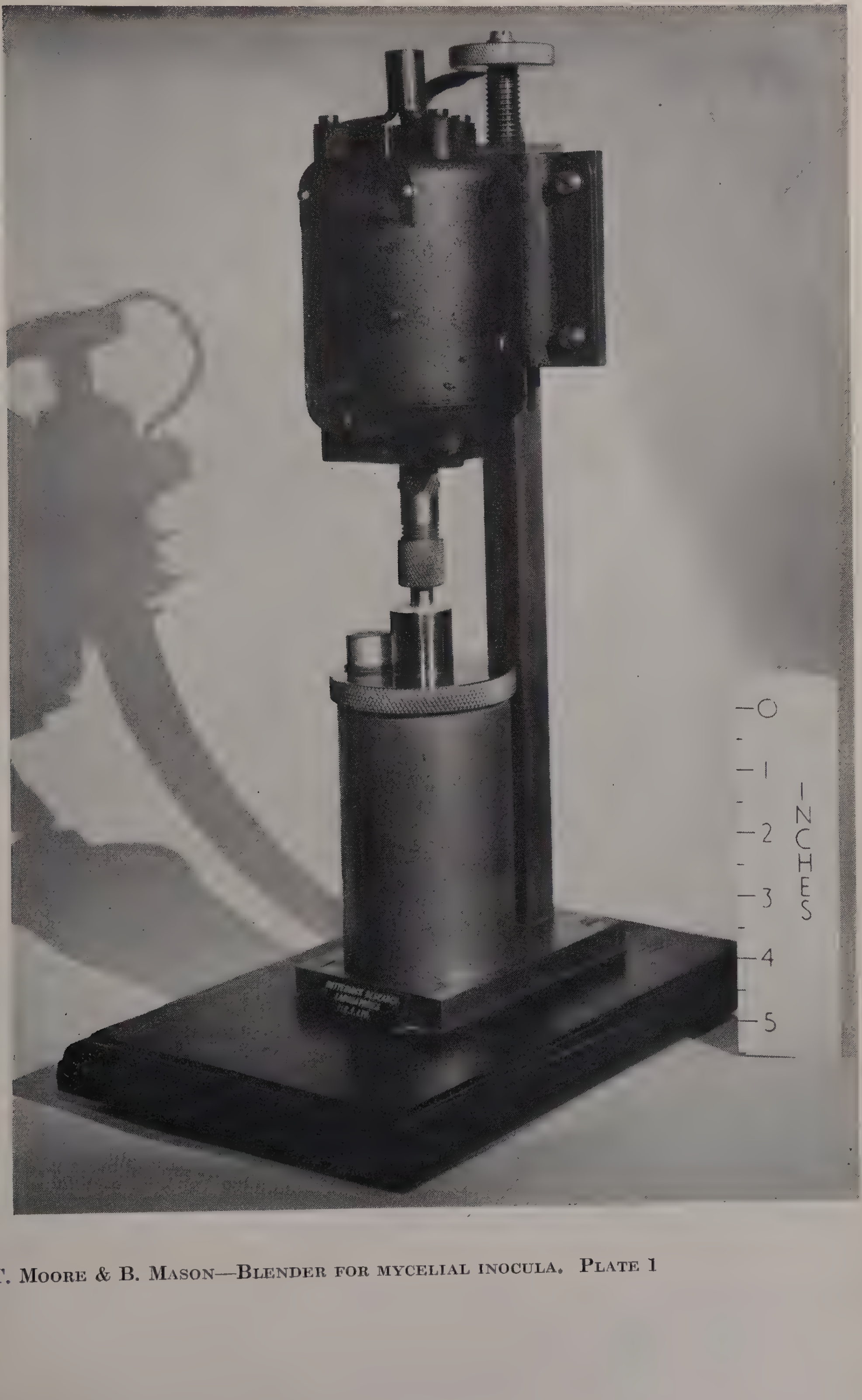

W. T. Moore & B. Mason—Blender for mycelial inocula. Plate 1

GRACE, JOYCE B. (1951). *J. gen. Microbiol.* 5, 519–524.

The Life Cycle of *Sporocytophaga*

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SUMMARY: The life cycle in *Sporocytophaga* closely resembles that of the non-sporing eubacteria.

The haploid microcyst germinates, the resulting cell containing two paired chromosomes reproducing vegetatively. An autogamous fusion and subsequent reduction division precede the maturation of the microcyst.

Sporocytophaga differs from *Myxococcus* by the absence of fruiting body formation, and in the details of its nuclear cycle. These bacteria may possibly be regarded as the ancestors of the higher myxobacteria.

The occurrence of a complex life cycle in the cellulose-decomposing cytophagas, which form large microcysts (*Sporocytophaga*, Stanier, 1942), is well known. It was first recognized by Hutchinson & Clayton (1919), who described the nuclear changes that occurred during the transformation of the rod-shaped bacteria into the large 'cocci' observed by earlier workers on decomposing cellulose fibres (van Iterson, 1904; Merker, 1911). Hutchinson & Clayton recognized that the 'cocci' were the resting stage and termed them sporoids, but did not observe their germination. Their preparations were dried and fixed by heat. This method distorts the vegetative cells, so that they appear as twisted, spiral rods, an appearance that misled these authors into believing that they were dealing with a spirochaete.

Bokor (1930) described the vegetative cells as long filaments which broke up into short rods, and finally divided into spores by fragmentation. This process is characteristic of many actinomycetes and, from his descriptions, it appears that he was dealing with mixed cultures containing one of these microorganisms. Both Bokor and Winogradsky (1929) considered that the 'sporoids' were contaminants.

Using more suitable cultural and staining techniques, Krzemieniewska (1930) confirmed most of the observations of Hutchinson & Clayton. She recognized the resemblance to the life cycle in myxobacteria, and also realized that the sporoids were microcysts. She described the nuclear changes during the germination of the microcysts, and observed an autogamous fusion preceding their maturation. Winogradsky (1935) later acknowledged the correctness of this interpretation of the life cycle.

MATERIAL AND METHODS

Fourteen strains of *S. myxococcoides* freshly isolated from soils and composts were examined. They were cultivated at 28° on filter-paper or cotton-wool moistened with a mineral salt solution containing 0·5 % NaCl; 0·1 % $M_9SO_4 . 7H_2O$, 0·1 % KNO_3 and 0·1 % K_2HPO_4 dissolved in tap water and the pH adjusted to 7·2.

Preparations were mounted in water and were not fixed, because it was observed that fixation by sublimate alcohol or osmic acid caused the vegetative cells to shrink considerably. The chromatin arrangement was identical in fixed and in unfixed preparations.

The chromatin material of eleven strains was demonstrable at all stages by staining with dilute aqueous Giemsa, provided that the dilution and time of application of the stain were adjusted to the individual strain. The vegetative cells of the remaining three strains could not be stained suitably by this technique; for these the acid-Giemsa technique (Robinow, 1942) was used.

Confirmation of the life cycle was obtained by the observations of living cells growing on agar containing finely divided cellulose. The cellulose was prepared by dissolving filter-paper in the minimum amount of concentrated sulphuric acid, and then precipitating it from this solution by rapid dilution with water; after washing to remove excess acid, it was added to a 2% agar containing the mineral salts.

OBSERVATIONS

The mature microcyst. The mature microcyst is found in culture about 2 weeks' old. It is a spherical structure, *c.* 2μ in diameter, containing a small, central mass of chromatin. The outer layer of the cell stains deeply (Figs. 1*a*, 2*a*).

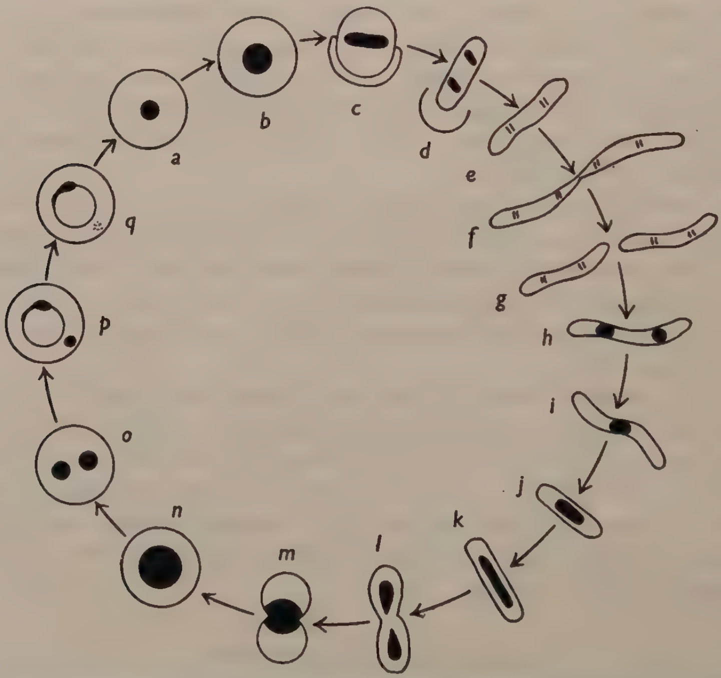

Fig. 1. Diagrammatic representation of the life cycle of *Sporocytophaga*. *a*, mature microcyst; *b-d*, germination of the microcyst; *e-g*, vegetative reproduction; *h-q*, microcyst formation.

Germination of the microcyst. When transplanted on to fresh medium, germination usually begins after a lag period of about 8 hr. The central mass of chromatin enlarges and is transformed into a thick, central bar. The cell shrinks slightly and begins to elongate, and the chromatin bar divides. At the same time, the envelope of the cell splits and is discarded (Figs. 1*a–d*, 2*b*, c). As the cell continues to elongate, the chromatin bars migrate towards the poles of the cell and are transformed into the paired chromatinic bodies, characteristic of the vegetative cell (Figs. 1*d*, *e*, 3*a*).

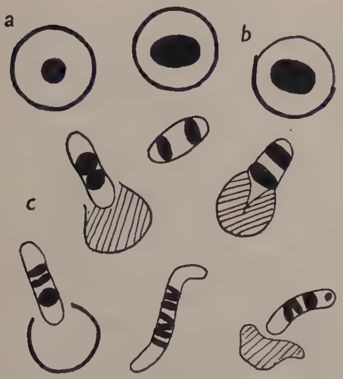

Fig. 2

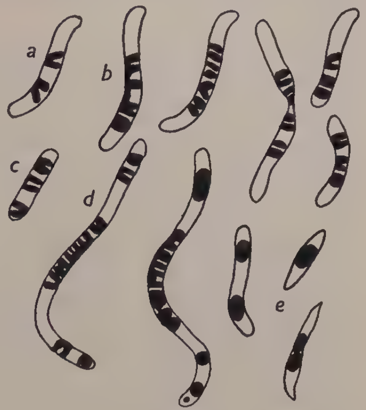

Fig. 3

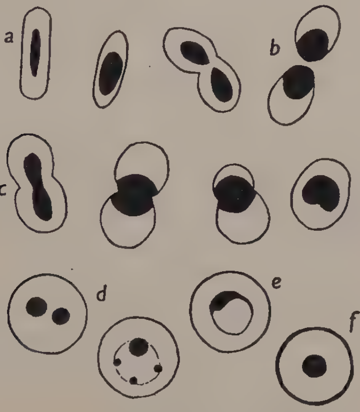

Fig. 4

Figs. 2–4. Drawings from Giemsa-stained preparations of cells of *Sporocytophaga*. Fig. 2. Germinating microcysts of *Sporocytophaga*. *a*, mature microcyst; *b*, first stage in the germination of the microcyst; *c*, liberation of the vegetative cell from the microcyst envelope. Fig. 3. Vegetative cells of *Sporocytophaga*. *a*, vegetative cell; *b*, stages in normal vegetative reproduction; *c*, trinucleate cell; *d*, filamentous cells; *e*, first stage in microcyst formation. Fig. 4. Stages in microcyst formation of *Sporocytophaga*. *a*, *b*, behaviour of chromatin material preceding fusion; *c*, nuclear fusion; *d*, reduction division; *e*, vesicular nucleus with tadpole-shaped chromatinic body; *f*, mature microcyst.

Vegetative reproduction. Normal living vegetative cells are weakly refractile rods, approximately $0{\cdot}3 \times 5\,\mu$. or more in size, with blunt rounded ends.

Spindle-shaped or spiral cells are only seen in stained preparations, especially those which have been fixed by heat or allowed to dry (Fig. 3*e*).

Ordinary vegetative reproduction is by fission. Each of the four chromosomes divides longitudinally, the cell begins to elongate and to constrict at the centre until two daughter cells are formed, each containing two pairs of chromosomes (Figs. 1*e–g*, 3*b*).

Filamentous cells were often observed in young cultures, but in most preparations it was impossible to observe the exact chromosomal arrangement. Preparations made from young cultures of all strains, however, showed cells containing three pairs of chromosomes, and in a few preparations, cells containing six and twelve pairs were observed (Fig. 3*c*, *d*). It seems probable, therefore, that the secondary method of vegetative reproduction, described in the non-sporing eubacteria by Bisset (1948), also occurs in *Sporocytophaga*. In this process the three chromosome pairs fuse at the centre of an enlarged cell. After two nuclear divisions, the chromatinic material is distributed throughout the growing filament, which finally fragments into six typical vegetative cells.

Microcyst formation. Microcyst formation may begin after 2–8 days, the exact time depending on the individual strain. The cell becomes shorter and the chromosomes are transformed into a central chromatin mass (Figs. 1*g–j*, 3*e*). The whole cell then enlarges slightly and the chromatinic material elongates and divides. This process is accompanied by a central constriction of the cell, which sometimes progresses to such an extent that two daughter cells are formed (Figs. 1*k*, *l*, 4*a*, *b*). The chromatin bodies then fuse again at the centre of the constricted cell, which becomes spherical (Figs. 1*m*, *n*, 4*c*). The fusion nucleus usually divides into two, but occasionally four bodies of equal size are formed. One (or, in the latter case, three) of these bodies gradually disappears. The remaining one is transformed into a large vesicular nucleus. The chromatinic portion of the latter takes the form of a body which appears to be shaped like a tadpole, but this appearance may be an optical illusion (Figs. 1*o–q*, 4*d*, *e*). Later this chromatinic material contracts into the small central body characteristic of the mature microcyst. The outer layer of the cell thickens during the latter stages of microcyst formation and begins to stain deeply.

DISCUSSION

The life cycle of *Sporocytophaga* appears to be as follows. The envelope of the microcyst is shed on germination. This process can be observed both in living cells, and in stained preparations as shown also by Krzemieniewska (1930), and Stanier (1942).

The vegetative cell contains two paired chromosomes, similar both in appearance and behaviour to those found in most Gram-negative bacteria (Bisset, 1950*a*). The diffuse distribution of the chromatinic material, described by Hutchinson & Clayton (1919), and by Krzemieniewska (1930), is only apparent in unhydrolysed preparations of those strains which cannot be stained directly with diluted Giemsa.

The maturation of the microcyst is preceded by a process suggestive of nuclear fusion. Hutchinson & Clayton depicted the fusion of the nuclear material at the centre of the constricted cell, but they claimed that a complete cell division took place with the formation of two sporoids. They apparently mistook the nuclear fusion for a cell division.

Krzemieniewska did not observe a reduction division, although she claimed that some of the chromatin material was rejected during the formation of the vegetative cells. From my observations, it appears that the reduction division immediately follows the nuclear fusion. The mature microcyst, therefore, contains a simple, central, haploid nucleus. This condition appears to be general in bacterial resting cells (Bisset, 1950*a*).

Sporocytophaga has been classified in the Myxococcaceae (Stanier, 1942), which it resembles in its creeping motility, but it differs from the other members of this family (*Myxococcus*, *Chondrococcus*) by the absence of fruiting-body formation. The formation of microcysts, which Stanier considers to be the main point of resemblance, is shared by many apparently unrelated groups of bacteria (Bisset, 1949, 1950*a*). In the details of the nuclear cycle, moreover, *Sporocytophaga* more closely resembles the non-sporing eubacteria than the myxococci (Klieneberger-Nobel, 1947).

The tendency to develop from an aquatic to a terrestrial mode of existence can be observed in bacteria (Bisset, 1950*b*), and an interesting evolutionary development can be seen in the Myxobacteriales. These bacteria are well adapted to a terrestrial existence by their creeping type of motility which requires a mechanical support. The highest evolutionary development in this group can be seen in the myxobacteria where the microcysts are grouped into fruiting bodies. In *Sporocytophaga*, the microcysts, although very resistant to drying and inanition, are formed and distributed singly. It is possible, therefore, that they may be the ancestors of the higher myxobacteria.

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KLIENEBERGER-NOBEL, E. (1951). *J. gen. Microbiol.* 5, 525–530.

The L-cycle: a Process of Regeneration in Bacteria

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SUMMARY: From a study of the mode of production of the L-phase from the bacillary phase and of the reversion of the L-phase into bacilli, by phase-contrast microscopy and in stained preparations, it appears that the L-cycle is a process of regeneration in bacteria.

Previous work (Klieneberger-Nobel, 1949*a*) on the L-phase in bacterial cultures was based on fixed and stained material. The phase-contrast microscope has made it possible to see significant detail in the living cell; the evidence presented in this paper for the existence of an L-cycle is based on a study by both methods.

METHODS

The organisms used were: the Morax-Axenfeld bacillus strain '260', *Proteus vulgaris, Shigella shigae,* strain 'KIR' and the stabilized L-phase of *Streptobacillus moniliformis* and *Fusiformis necrophorus,* L 1 'An' and L 'Fair' respectively, both isolated by me in 1949.

For the study of the living material nutrient agar plates were inoculated and cut into pieces 0·75 cm. square, the inoculated surface was covered with a sterile cover-slip and the whole sealed to slides with paraffin wax. These preparations were examined with a phase microscope fitted with a long-distance condenser; usually at a temperature of 32°, but sometimes room temperature and 4°. Preparations were fixed and stained by the author's Bouin Giemsa method and Robinow's method for nuclear structures (Klieneberger-Nobel, 1950).

OBSERVATIONS

Morax-Axenfeld bacillus, strain 260

The strain grew well on serum agar, producing long chains of bacilli. It was always pleomorphic, and produced L-forms freely. However, the production of L-forms was greatly enhanced by the addition of 0·05–0·07 % of glycine (Gordon & Gordon, 1943) to the serum agar medium at pH 7·8–8·0. During the first 2 hr. at 32° no observable change occurs except growth. Soon afterwards the habit of the cultures begins to change; many filaments composed of chains of organisms bend sharply so that the two limbs of the bend lie alongside one another; twisting begins and continues until double spirals are produced. The twisting of a number of filaments can be followed in consecutive photographs (Pls. 1 and 2, figs. 1–10), taken at intervals of 25 min. The topmost filament, out of focus in the first figure, but in focus in the second one, started to twist (Pl. 1, fig. 2) and other bent filaments (Pls. 1 and 2, figs. 3–8) did likewise. In the next stage after 5 hr. 25 min. the longest of the double spirals became deformed and started to break up (Pl. 2, fig. 9). Twenty-five minutes later the contents of the cells flowed out of their boundaries and spread over the agar surface (Pl. 2, fig. 10). I assume that at this stage cell elements unite with each other and that the row of soft, shapeless forms which replace the double spiral is a chain of L-bodies. This peculiar mode of L-form production in Morax-Axenfeld 260 is seen in a further developed stage in Pl. 2, fig. 12. The illustration shows large single bodies separated from each other, but showing by their arrangement their origin from twists. Twisting does not always precede L-form production. Sometimes the production of loops, convolutions or skeins seems to serve the same purpose, bringing different filaments into close contact with each other, so that fusion of cell elements can take place. Before the appearance of L-forms small granular elements invariably develop, which are seen within the bacterial filament or become free. Granules are also often seen sticking to filaments. In Pl. 2, fig. 11 and Pl. 3, fig. 13, a number of such granules can be detected in two similar microscopical fields from a phase contrast and stained preparation respectively. Some of the L-bodies here already developed have probably not formed from double spirals but may have been produced by fusion of granular elements. Further research will be necessary to clear up the actual mode of fusion, but I think it will be found that small elements play a most important part in this process.

As incubation proceeds whole areas of growth are transformed into L-elements (Pl. 3, fig. 14); but after another 12 to 18 hr. the L-bodies revert to bacilli as in Pl. 3, fig. 15, where traces of the loose open pattern of the previous L-growth can still be detected underneath the bacillary filaments. Granules belonging to the L-phase are also present.

Shigella shigae *strain KIR*

The reversion from L-phase to bacillary form has also been studied in this rough *Shigella* strain. In Pl. 3, fig. 16, the bacillus is completely in the L-form, and fig. 17 shows the bacillary chains derived from the L-form after another 12 hr. incubation. Here, too, the open lacy pattern of the original L-growth can still be discerned.

Proteus vulgaris

P. vulgaris readily goes into the L-stage when the temperature is lowered appreciably. Preparations were made of the swarming edge of 15–18 hr. cultures on nutrient agar. When the cultures were cooled to 4° some of the long filamentous swarmers (Pl. 3, fig. 18), of which the edge of the growth consisted, started a new development. They became less dense, and looked 'faded' both under the phase microscope and after staining. Eventually these filaments broke up into small granular elements (Pl. 3, figs. 19, 20). After 1 hr. in the cold there were many granules (Pl. 4, fig. 21) which increased in numbers the longer the cultures remained in the cold. The granules are indeed remarkable, and it should be noted that the diameter of the smallest of them is much less than the width of the ordinary proteus cell.

As has been previously suggested (Klieneberger-Nobel, 1949*a*) two or perhaps more of the small elements may fuse, although the process has not yet been observed. However, at this stage the shapes of the elements are so suggestive that I am convinced that fusion may be a justifiable interpretation. For example, in the upper part of Pl. 4, fig. 22, twisted filaments can be seen which look very much like the precursors of the 'fused twists' in the lower part of the same illustration. Suggestive are also the small formations indicated by the arrows in Pl. 4, fig. 21, which are seen between the dark bacillary filaments. Like the granules they are lying on the faded bacillary filaments, but they are larger and some of them are dark, others are pale. From observations it is known that they are rapidly going to develop into L-bodies.

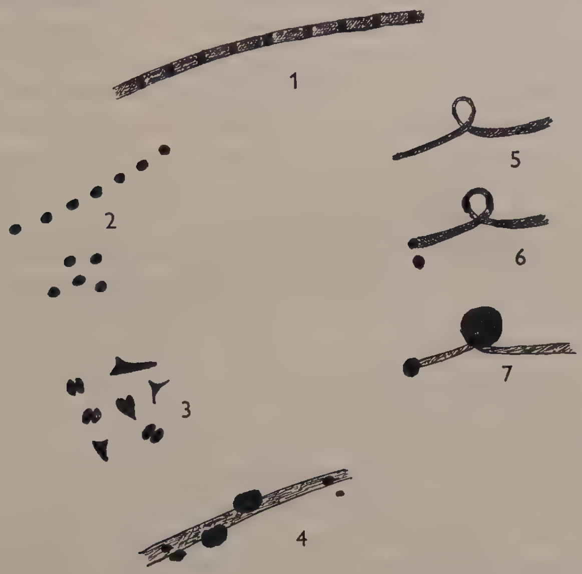

Fig. 1. Schematic diagram of the formation of L-bodies from bacillary forms.

In Fig. 1 the hypothetical formation of L-bodies is illustrated. A bacillary chain (1) breaks up into granules (1 and 2). These fuse (3) and develop into L-bodies either free or in connexion with a bacillus (4) or the bacillary chain forms a loop (5) which produces granules in the loop or at the tip of the filament (6), which may develop into bodies (7). As seen in Pl. 4, figs. 23 and 24, most of the developing bodies are lying free, but if they are connected with bacilli they are either situated at the very tips where previously granules were observed or they are found on the inside of the curved bacillary filaments where they were probably produced by loops. Of course, if they grow up from granules which stuck to a bacillus they may well simulate swollen bacilli.

With longer exposure to cold more granular forms, and bodies of various sizes which grow from the presumably fused granules, appear. Pl. 4, fig. 23, illustrates a typical 'swarming edge' that was kept in the cold overnight. The bodies are dense in these early stages, and some of them stand out almost jet-black under the phase microscope. A similar field from a stained specimen is seen in Pl. 4, fig. 24. Apart from granules and bodies of various sizes the darkly stained normal bacillary filaments and the faded filaments which give rise to more and more granules can be well distinguished in this illustration.

When at the stage depicted in Pl. 4, figs. 23 and 24 the cultures are re-incubated at 32° the still untransformed swarmers become vigorously motile. This proves that the cultures have not been killed by the cold; they form a new edge by 'swarming' according to their peculiar and most fascinating pattern. The granules and bodies immediately increase in size and decrease in density. These bodies are readily deformed when pushed by an actively motile swarmer indicating that they are very soft; they have no motility of their own. After a few hours, some of the now very big bodies (Pl. 4, fig. 25, phase contrast) divide into smaller ones by a process of segmentation. These latter bodies may grow up again and form small groups consisting entirely of L-elements which shows that they are able to grow and multiply independently. When incubation is further continued, the L-bodies form points and tapers and develop into peculiar often amoeboid shapes. At the same time they become denser and start on a process of continuous subdivision into smaller and thinner forms (Pl. 4, figs. 26 and 27, phase contrast). The L-forms are on their way to transform into bacilli. At this stage 'branching' is very often simulated as illustrated in Pl. 5, fig. 28 (stained preparation). The same phenomenon has been illustrated and described by Fleming, Voureka, Kramer & Hughes (1950). Bacillary endings of complex forms (indicated by arrow in fig. 28) become motile again, and if they still carry about a globular part they may present a most fantastic sight. When the subdivision and transformation is complete the short young forms and not the long, swarming filaments are produced (Klieneberger-Nobel, 1947). Thus the life cycle is complete; the young forms produce the swarming cells after 3–4 hr. on a new medium. The swarmers, if exposed to the cold, produce the L-forms by breaking up into small elements which after a process of presumed fusion grow up into the big bodies. The L-form is capable of independent multiplication, but usually reverts into the bacillary stage.

DISCUSSION

From the material presented there is indication that L-phase production is often preceded by special characteristic configurations of the elements of the culture. One particular way is the formation of double spirals which brings two different cells into juxtaposition. This occurred frequently in the Morax-Axenfeld and in *Streptobacillus moniliformis* and sometimes in *Proteus vulgaris* and other Gram-negative bacteria. Developments also occurring at this stage are formation of loops, convolutions and skeins. It is postulated that the appearance of these growth forms is followed by a fusion of small constituent elements which thus have been brought together. Whenever these configurations were observed, L-forms arose soon afterwards in their place, and at the same time the double spirals, loops and convolutions disappeared. Yet there seems to be another way of bringing together the small elements for fusion; they may be freed when the mother filaments fade and disintegrate. This happened to a large extent in *P. vulgaris*. The L-forms produced from looping or twisting filaments are therefore more often found in connexion with the bacilli, while those produced from disintegrating filaments are more often observed independently. More work will be necessary to unravel completely the mode of L-form production. However, the material presented will, it is hoped, give direction to future investigations.

Some organisms go through the L-cycle and revert into the bacterial stage each time they grow. *S. moniliformis* and *Fusiformis necrophorus* belong to this group of microbes. Slightly adverse conditions seem to stimulate other organisms to go through their L-cycle (Klieneberger-Nobel, 1951). These stimuli retard the multiplication rate at a state of vigorous growth, creating a disturbance to which the organism reacts by transforming into the L-phase, in which it can withstand the adverse conditions. When the bacterial phase is newly produced from the L-phase it is often found that new races have arisen better adapted to the adverse conditions than the mother organism. The L-cycle may therefore be regarded as a process of regeneration, initiated by a fusion of certain elements.

The bacillary cells differ from the L-stage in the following respects. The bacterium has a rigid form, and the elements of the L-phase are soft and consequently vary widely in shapes and sizes; Pl. 5, figs. 29, 30, show the stabilized L-forms of *F. necrophorus* (phase contrast) and *S. moniliformis* (stained) respectively. Secondly, bacteria divide by binary fission, and the L-bodies multiply by segmentation and eventually break up into small granules which germinate again and which in *S. moniliformis* are filterable through membranes that retain the smallest bacteria (Klieneberger-Nobel, 1949*b*). The L-stage also differs from the bacterial stage in respect of food requirements and metabolism. There are serological differences and very possibly great differences in regard to pathogenicity.

I should like to emphasize that the organization of the L-phase is similar in many important aspects to that of the pleuropneumonia-like organisms. For here we find the same shapelessness and softness and most important, the breaking into fertile granular elements of approximately the same size and filterability.

There have been many investigators who postulated a complex life cycle and reported similar observations and offered similar interpretations. I have discussed their work and the pertinent literature in a recent review (Klieneberger-Nobel, 1951).

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EXPLANATION OF PLATES

Figs. 1–12, 14–19, 23, 25–27, 29. Phase contrast.
Figs. 13, 20–22, 24, 28, 30. Fixed and stained.

(Magnification × 2200 throughout.)

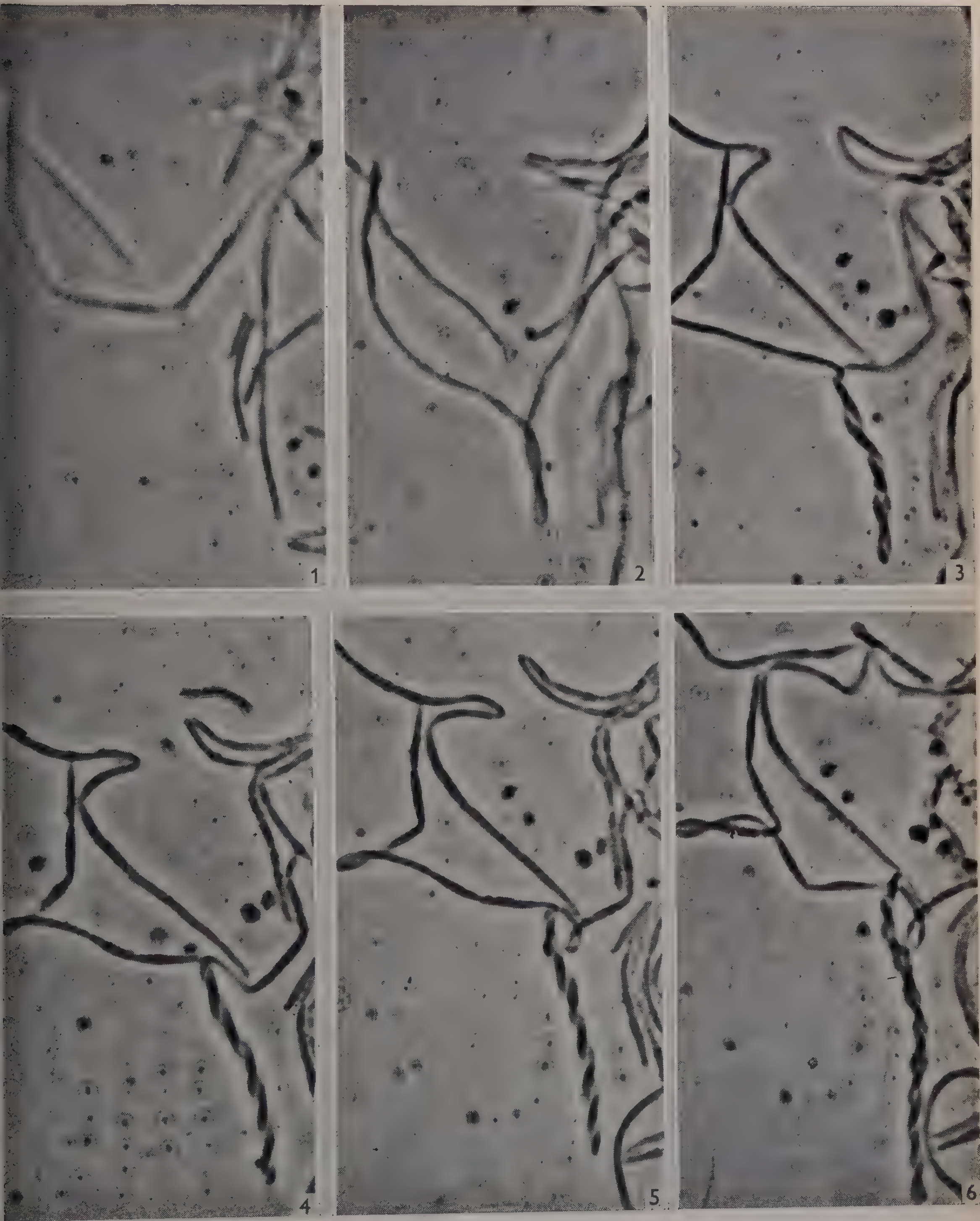
Figs. 1–15. Morax-Axenfeld bacillus on serum agar plus 0·05–0·07 % of glycine. Figs. 1–10. The production of L-bodies by double spirals. Fig. 1 was taken after 2 hr. of incubation; the subsequent photographs were taken at intervals of 25 min. Fig. 11. L-bodies and granules on filaments. Fig. 12. Double spirals transforming into L-bodies. Fig. 13. L-bodies and granules stained. Fig. 14. Groups of L-bodies which had developed after 24 hr. of incubation. Fig. 15. After several days' incubation, long bacillary filaments have arisen from L-growth.

Figs. 16, 17. *Sh. shigae* 'KIR' on serum agar plus 0·4 % glycine. Fig. 16. After 7½ hr. at 32° and 13 hr. at room temperature. The photographed area shows L-growth exclusively. Fig. 17. The culture in fig. 16 after additional incubation at 32° for 6 hr. Bacillary filaments have redeveloped.

Figs. 18–28. *P. vulgaris* on nutrient agar. Fig. 18. Swarming edge. Fig. 19. Part of swarming edge kept in the cold for 4 hr. A broken-up filament with granules is seen. Fig. 20. Part of swarming edge kept in the cold for 1½ hr. Bacillary filaments broken up into granules are seen. Robinow's staining method for nuclear structures. Fig. 21. Part of swarming edge kept in the cold for 1½ hr. Notice the granular elements. Fixed with osmic acid vapours and stained with Giemsa solution. Fig. 22. Swarming edge after 1½ hr. in the cold. In the upper part of the photograph a few double spirals are seen which correspond to 'fused double spirals' in the lower part of the field. Bouin Giemsa method. Fig. 23. Swarming edge kept in the cold overnight. Fig. 24. Swarming edge kept in the cold overnight. Bouin Giemsa preparation. Fig. 25. Swarming edge kept in the cold overnight and reincubated for 3 hr. The L-bodies have grown considerably and have become irregular. Fig. 26. The same as fig. 25, but 4 hr. of reincubation. Subdivision of the bodies, which are on their way to reproduce bacilli, is progressing. Fig. 27. The same as figs. 25 and 26, but 6 hr. of reincubation. Subdivision and redevelopment of bacteria has still further progressed. Fig. 28. Redevelopment of bacteria from L-forms. At the bottom is seen a huge branched form. At the top young bacilli are seen in a group. Bouin Giemsa preparation.

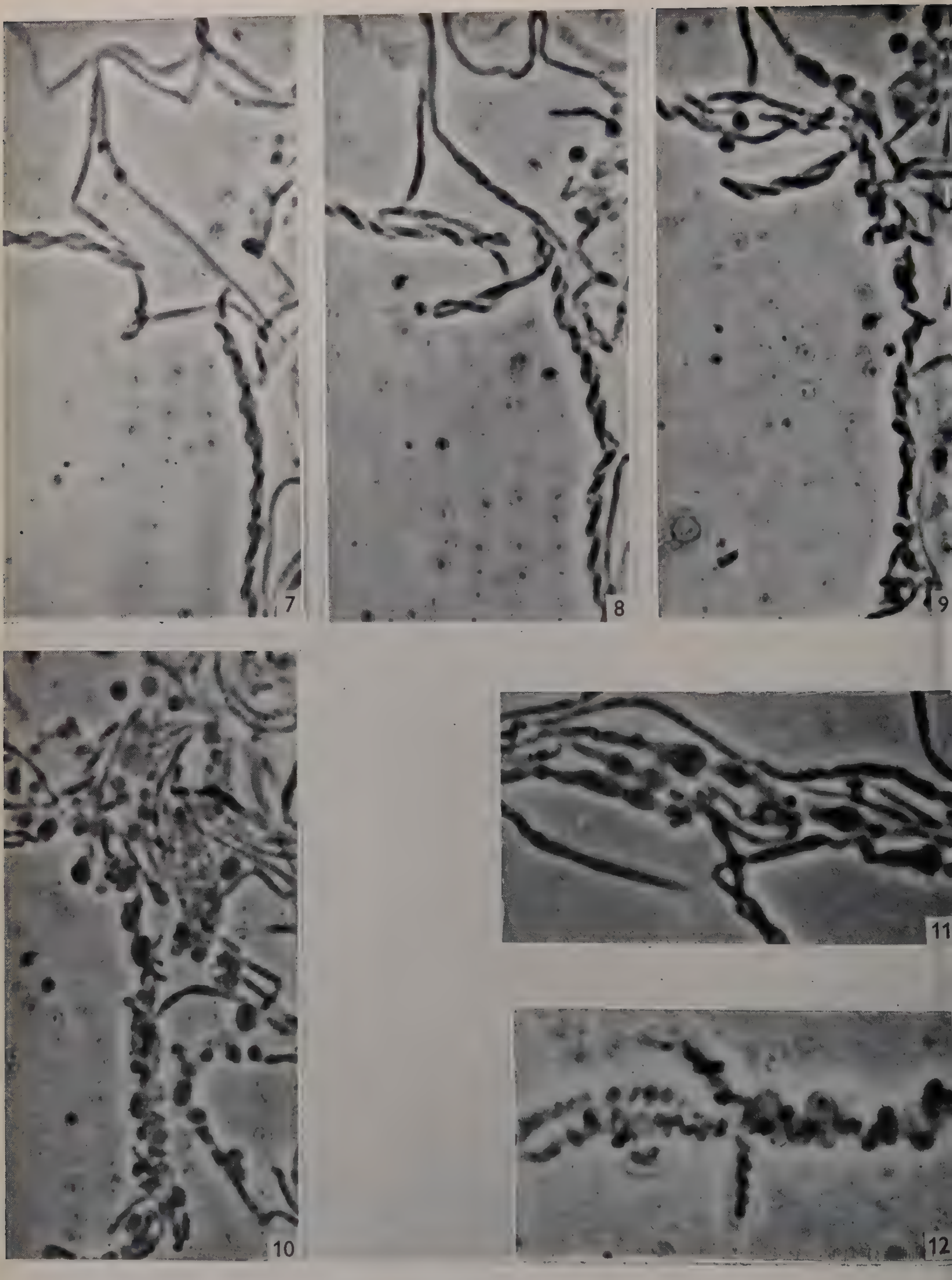
Fig. 29. L 'Fair' (pure L strain from *F. necrophorus* isolated in 1949) grown in semi-solid medium for 3 days. Bodies and droplets of a cholesterol-like material are to be seen. Fig. 30. L 1 'An' (pure L strain from *S. moniliformis* isolated in 1949) grown on serum agar for 2 days. Large amoeboid forms containing granular matter are to be seen. Bouin Giemsa preparation.

(*Received* 2 *January* 1951)



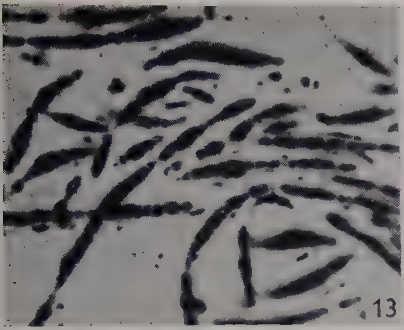
Figs. 1–6

E. KLIENEBERGER-NOBEL—THE L-CYCLE IN BACTERIA. PLATE 1



Figs. 7–12

E. Klieneberger-Nobel—The L-cycle in bacteria. Plate 2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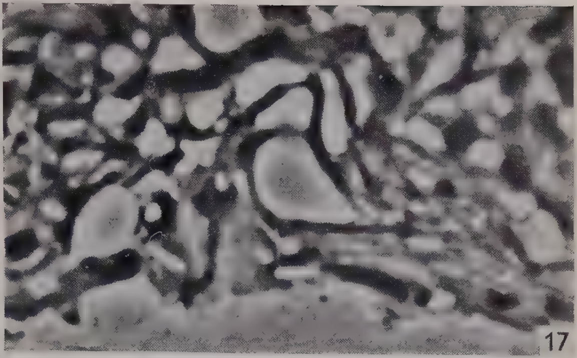
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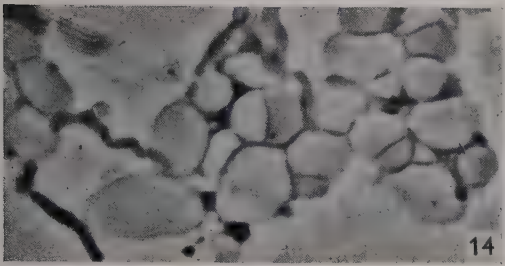
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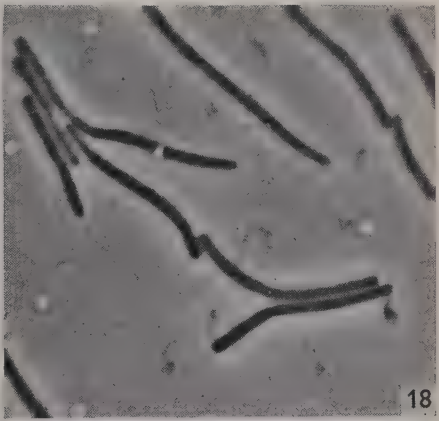
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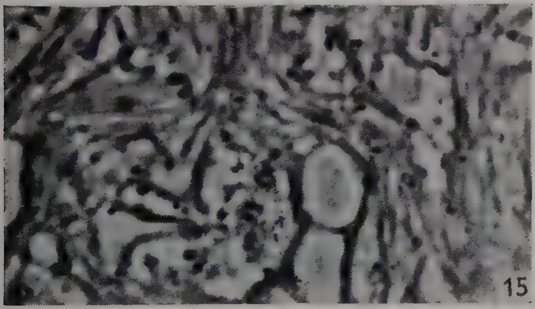
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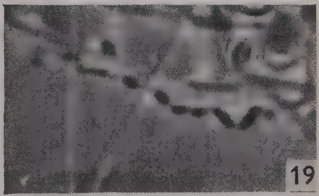
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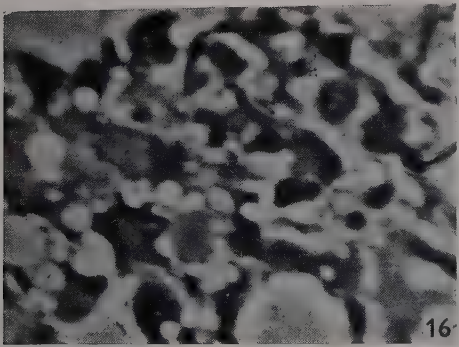
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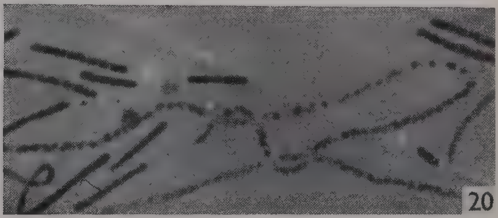
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Figs. 13–20

E. Klieneberger-Nobel—The L-cycle in bacteria. Plate 3

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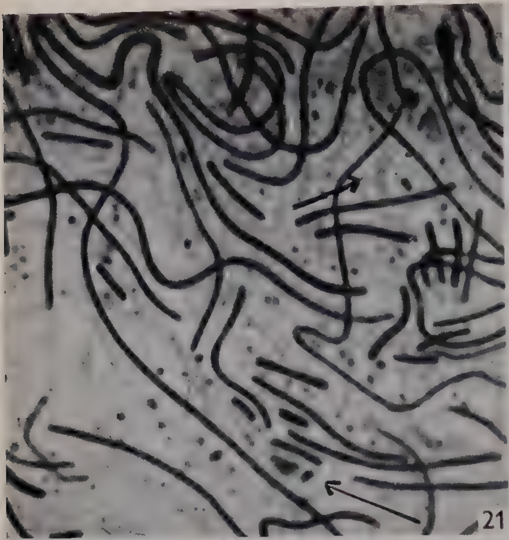
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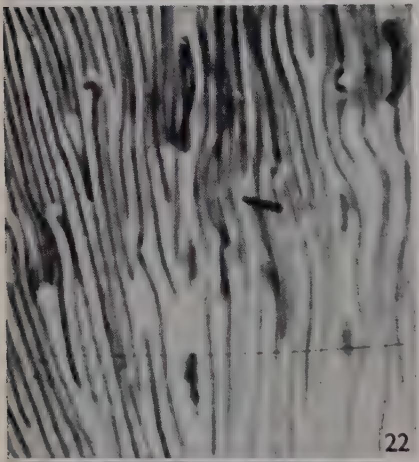
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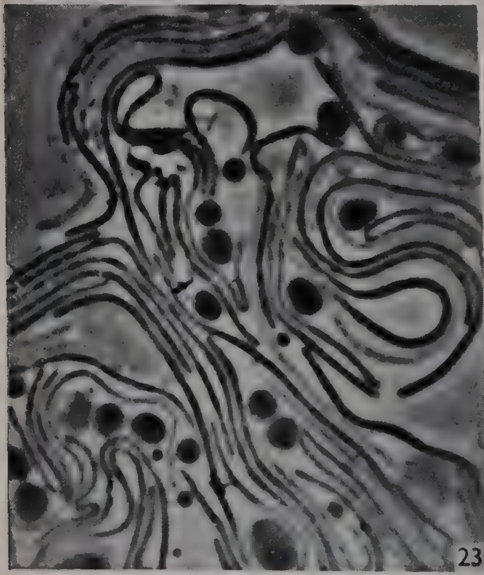
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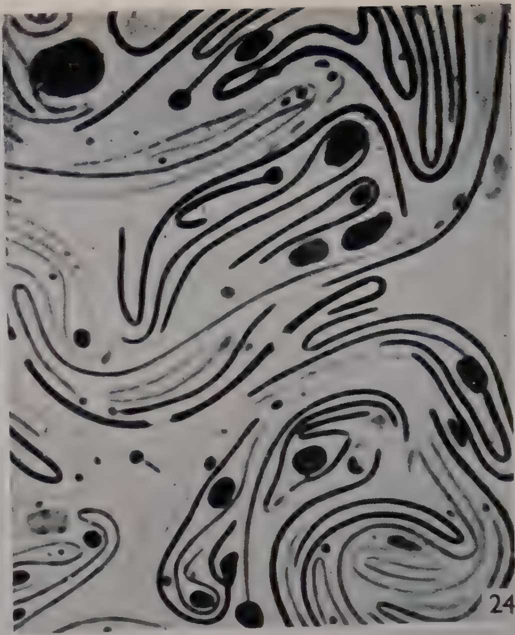
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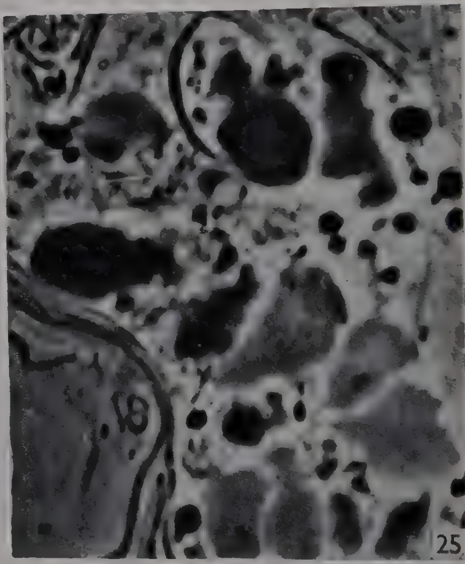
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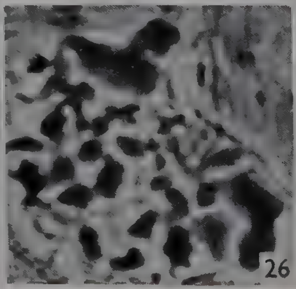
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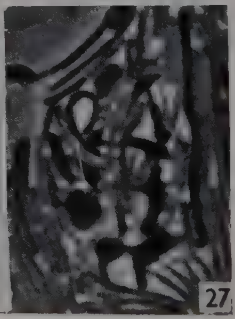
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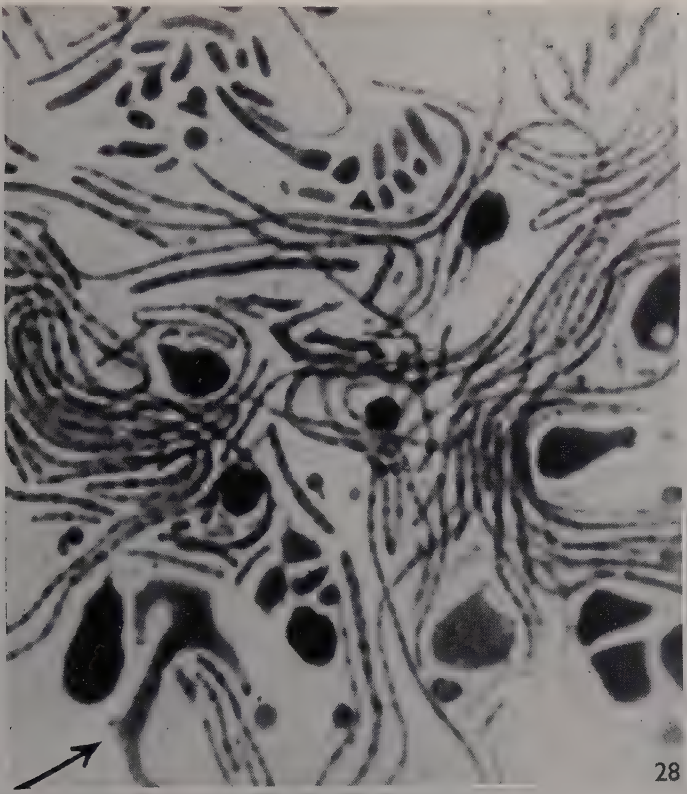
Figs. 21–27

E. Klieneberger-Nobel—The L-cycle in bacteria. Plate 4



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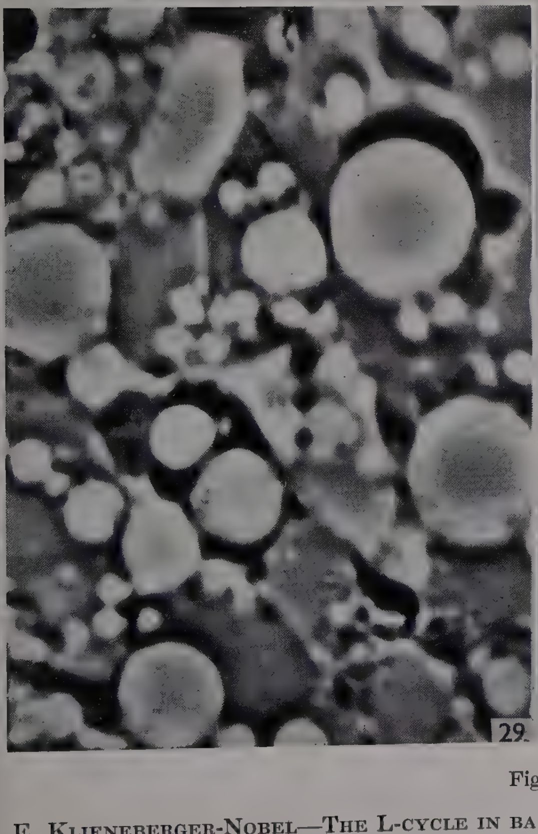

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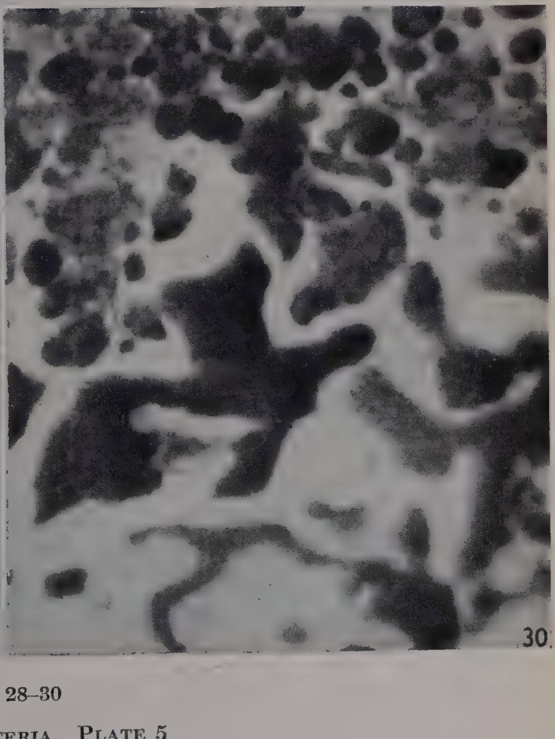

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Figs. 28–30

E. Klieneberger-Nobel—The L-cycle in bacteria. Plate 5

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The Phase I of *Haemophilus pertussis*

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SUMMARY: An examination of freshly isolated *Haemophilus pertussis* strains for agglutinability, growth requirements, haemagglutination, virulence for mice, alum precipitability and protective properties shows that each of these characters varies quantitatively, and furthermore that they vary independently. The presence of one strongly marked character is no indication that another character will be strongly marked or even present. Not all strains necessarily have the characters to be expected in freshly isolated strains; one or possibly more may be missing. On subculture of the strains on Bordet-Gengou media the order in which these characters degenerate or are lost and the rate at which they are lost differs with the strain. Agglutinability and agglutinogen content vary considerably in freshly isolated strains, and therefore the expression 'agglutinates to titre' cannot be considered as a criterion of a Phase I organism. The obvious complexity of the serology of organisms within 'Phase I' suggests that this term has served its useful purpose and should now be dropped as a description of a certain type of strain. The term '*Smooth*', which indicates the possession of the antigenic components commonly associated with the pathogenic state of the organism, is preferable.

Leslie & Gardner (1931) analysed thirty-two strains of *Haemophilus pertussis* by agglutination and agglutinin absorption and found that they fell into four well-marked groups which they called Phases I, II, III and IV. They suggested that Phases I and II probably correspond to the smooth (S) and Phases III and IV to the rough (R) phase of other bacteria. This work was confirmed by Lawson (1939) with respect to Phases I, III and IV but he did not find any strains in Phase II. He stated, however, that 'there are undoubtedly other intermediate stages in the dissociation process, their number dependent on the strain of the organism and its environmental condition', and suggested that the degradation proceeds gradually, often making it difficult to determine the border-line between the phases.

The investigation of *H. pertussis* has tended to proceed along one of two lines; either the investigation of the agglutinogenic relationship of the phases, as made by Flosdorf, Dozois & Kimball (1941), who presented evidence that variation is even more complex in minor relationships than former workers had found, or the characterization of Phase I organisms in the hope of finding a single character which would clearly distinguish organisms in this phase.

What then is Phase I, and are all organisms in Phase I identical? Historically Phase I was defined serologically, but recent work (Smolens, McLaren, McAleer & Flavell, 1947; Kendrick, Updyke & Eldering, 1949; Cohen, 1949) has shown that freshly isolated strains may vary considerably in their agglutinogen content, and that a small number of typical strains are of consistently low agglutinability. Leslie & Gardner implied, though they did not state it, that Phase I organisms only grow on Bordet-Gengou medium while Phases III and IV will grow on agar.

Lawson (1939) tabulated other characters, by which he distinguished the main phases, and later workers added more characters usually differentiating even more clearly Phase I from Phase IV; e.g. Burnet & Timmins (1937) used virulence for mice by the intranasal route, Kendrick *et al.* (1949) virulence for mice by the intracerebral route, Keogh & North (1948) haemagglutinin, Ungar & Muggleton (1949) alum precipitability and bile solubility. These investigations, while they have added to our knowledge of Phase I, have not made it any easier to define. Usually the workers were interested in the selection of strains optimal for some such purpose as vaccine manufacture, and have implied or even stated that the presence or assay of one character in a strain can be taken as a measure of another character (e.g. Smolens *et al.*, 1947, Keogh & North, 1948, Ungar & Muggleton, 1949). My investigation (Standfast, 1951) of the virulence for mice by the intranasal route and its correlation with certain other factors showed that when a large number of strains were examined there was little if any correlation between some of the characters of freshly isolated *H. pertussis*. It was felt that a re-examination of the main characters of Phase I was worth while in the hope that a more certain definition of Phase I might emerge. The characters used by most workers for differentiating strains are: (*a*) growth requirements, (*b*) agglutination and agglutinin absorption, (*c*) virulence, (*d*) protective antigenicity, (*e*) haemagglutinin, (*f*) alum precipitation and bile solubility, and to some extent (*g*) morphology. In the experiments described in this paper about 100 strains of *H. pertussis* freshly isolated from clinical cases of whooping cough were examined for these characters.

MATERIAL AND METHODS

Strains. Strains of *H. pertussis* were freshly isolated from patients with whooping cough, the first subculture from the original cough or swab plate being sent to the laboratory. All strains agglutinated with sera prepared against freshly isolated *H. pertussis* strains. They consisted of typical small Gram-negative cocco-bacilli. There was some variation in size from strain to strain, certain strains being smaller, $0{\cdot}5$–$0{\cdot}6\ \mu. \times 0{\cdot}3$–$0{\cdot}4\ \mu.$, whereas others measured $0{\cdot}7$–$0{\cdot}8\ \mu. \times 0{\cdot}4$–$0{\cdot}5\ \mu.$ This size variation was on the whole consistent in the strain, and though on some batches of Bordet-Gengou medium the 'smaller' strains grew rather 'larger', this increase in size was not permanent. There was no correlation between size and any other character. After prolonged subculture on laboratory media all strains tend to become larger; but the strains examined all remained cocco-bacilli and did not become 'coliform' in size even after subculture on blood agar.

Medium. Bordet-Gengou agar was used, containing 1 % Difco Proteose peptone (Baird and Tatlock Ltd., London) and 33 % horse blood. Nutrient agar was prepared from horse meat digest, to which 10 % horse blood was added to make blood agar.

A heavy inoculum on a bent glass rod was rubbed over half the Bordet-Gengou plate, then carried over to the whole of the blood plate, then the whole of the nutrient agar plate and then the second half of the Bordet-Gengou plate. In all cases both halves of the Bordet-Gengou plate showed confluent growth after 24 hr. at 35°, indicating that there was an adequate inoculum on the blood agar and nutrient agar plates. There was never any growth on the agar plates after 5 days at 35°. Any growth on a blood agar plate was subcultured on a second blood agar plate, and only if this grew was growth considered positive on blood agar. On several occasions though growth occurred on the first blood agar plate it was not obtained on the second, indicating perhaps that the growth on the first plate was due to essential metabolites carried over with the inoculum.

Agglutination test. All agglutination tests were carried out in tubes, incubated at 55° in a water-bath for 4 hr. and then stood at room temperature overnight. The titre is the reciprocal of the highest dilution which shows obvious agglutination with a ×3 hand-lens.

Sera were prepared in rabbits given three doses of 10^9 formalized bacilli subcutaneously followed by three doses of 10^9 living bacilli intravenously at weekly intervals.

Agglutinin absorptions were carried out by the method of Smolens *et al.* (1947) with certain modifications. A suspension of bacteria of any desired wet weight in a volume of 1 ml. were added to 1 ml. of a 1/250 dilution of antiserum, mixed and incubated for 2 hr. at 55° and then placed in the refrigerator overnight. Next morning the tubes were centrifuged and 0·5 ml. of dilutions of the supernatant were added to 0·5 ml. of the test suspension, incubated for 4 hr. at 55° and then stood at room temperature overnight. Serum controls were mixed with saline. The absorption titre was taken as the mg. wet weight of the strain required to absorb 90 % of the agglutinin.

Virulence tests. Intranasal virulence was titrated by the technique of Burnet & Timmins (1937). Briefly four doses of 100, 10, 1 and 0·1 million were each instilled intranasally into a group of ten mice kept under standardized conditions.

Intracerebral virulence was titrated by the method of Kendrick *et al.* (1949) using groups of ten mice on each dose. Mice of the strain Cl. used in these laboratories required doses about ten times greater than the strain of American mice, a few of which were available for comparative purposes.

The LD 50 of each strain was calculated by the method of Reed & Muench (1938).

Haemagglutinin tests. Suspensions for the haemagglutination test were prepared in saline or Difco Casamino acids (Baird and Tatlock Ltd., London) and standardized to contain 10^{10} organisms/ml., and serial twofold dilutions were prepared from these suspensions. Red blood corpuscles (R.B.C.) were prepared by centrifuging 1 ml. of fowl's blood, washing the R.B.C. three times in saline and resuspending in 50 ml. of saline. This 'strong' suspension can be kept 3 or 4 days in the refrigerator; before use it is diluted 1/4 with 0·85 % saline. It was found that 0·5 ml. of this dilute suspension was the optimum when added to 0·25 ml. of bacterial suspension.

For the test 0·25 ml. of the dilution of bacterial suspension was placed in a 12 × 55 mm. tube, and 0·5 ml. of R.B.C. suspension added. The racks were shaken and incubated at 37° for 1 hr. The tubes were then shaken and put in the refrigerator to allow the cells to settle before reading the result.

The titre is taken as the reciprocal of the final dilution of bacterial suspension which shows obvious agglutination of the R.B.C. by eye.

Protection tests. Vaccines, prepared on horse blood Bordet-Gengou agar, were killed with 0·2 % Liq. Formaldehyd B.P. (1948) diluted to 10^{10} bacilli/ml., and tested by the method of Kendrick, Eldering, Dixon & Misner (1947). The ImD 50 calculated by the method of Reed & Muench (1938).

Aluminium phosphate precipitation was carried out by the method of Ungar & Muggleton (1949). Three-tenths ml. of 5 % Na_2HPO_4 followed by 1 ml. of 2 % potassium alum was added to 4·0 ml. of suspension of 10^{10} bacilli/ml. in saline containing 0·2 % Liq. Formaldehyd, shaken and stood for 30 min. before reading.

EXPERIMENTAL

Growth requirements

On various occasions groups of strains were plated on Bordet-Gengou agar, blood agar and nutrient agar.

One strain M 7 grew abundantly on blood agar immediately after first isolation. Three cultures of this strain, all behaving in the same way, were obtained from a child in an outbreak of whooping cough in a children's ward. In all other respects this strain was typical (Table 1). This is the only strain in this series which grew, and grew profusely, on blood agar from first isolation.

Of the seventy-one strains tested, eight showed some growth on blood agar seeded with the 2nd, 3rd and 4th subculture on Bordet-Gengou. Viable counts

Table 1. *Modification of characters of four strains of* Haemophilus pertussis *which grew on blood agar, during subculture on Bordet-Gengou or blood agar*

Strain	Sub-culture growing on blood agar	Agglutination titre		Alum precipitability (%)		Haemagglu-tination titre		Protective antigen (ImD 50 × 10^9)		Virulence (intranasal) (LD 50 × 10^6)	
		N		N		N		N		N	
M 7	1	(3)	16,000	.	nt	(4)	0	.	nt	(3)	1
		[3]	16,000	.	nt	[4]	0	.	nt	[3]	1
		(17)	16,000	(17)	100	.	nt	(17)	0·20	(17)	1
		[17]	16,000	[17]	100	.	nt	[17]	0·21	[17]	27
C 5467	4	(9)	16,000	(9)	100	(2)	48	.	nt	(2)	35
		(4)+[5]	4,000	(4)+[5]	100	(4)+[5]	0	.	nt	(4)+[5]	40
C 315	6	(6)	16,000	.	nt	Always negative		(15)	4·0	(9)	5
		(41)	16,000	.	nt			.	nt	.	1
		(6)+[9]	4,000	.	nt	Always negative		(6)+[9]	5·0	(6)+[3]	4
		(6)+[19]	2,000	.	nt			.	nt	.	1
C 407	7	(15)	32,000	.	nt	(15)	12	(8)	0·85	(15)	0
		(32)	32,000	.	nt	(32)	12	.	nt	(32)	1
		(8)+[7]	16,000	.	nt	(8)+[7]	0	(8)+[7]	1·6	(8)+[7]	1
						(8)+[14]	0	.	nt	(8)+[14]	11

N, number of subculture. ( ), on Bordet-Gengou. [ ], on blood agar. (4)+[5], 5th serial subculture on blood following four subcultures on Bordet-Gengou. nt, not tested.

were made on Bordet-Gengou and blood agar on three of these strains. The ratio of colony counts on blood agar to Bordet-Gengou were 1:340 million, 1:940 million and 1:1270 million.

Stability of strains with respect to growth requirements on continued subculture

A group of twenty strains were plated daily on Bordet-Gengou agar and at intervals on blood agar. Five showed some growth on blood agar after 11th, 12th, 12th, 16th and 25th subcultures on Bordet-Gengou. The remaining fifteen strains did not grow on blood agar after sixty subcultures on Bordet-Gengou.

Another group of seventeen strains were plated daily on Bordet-Gengou agar for over 200 subcultures, and once a week on blood agar. Growth appeared on blood agar from seven of these strains after 38th, 114th, 190th, 197th, 200th, 203rd, and 211th subcultures on Bordet-Gengou. The other ten strains did not acquire the capacity to grow on blood agar.

The twelve strains mentioned above and the eight strains growing on blood agar soon after first isolation all produced from 10 to 100 isolated colonies on the first blood agar plate after 1–5 days at 35°. If these strains are identical with the strain isolated by Pollock (1947) it would seem that some (eight out of seventy-one) freshly isolated *H. pertussis* carry the variant. It may, however, arise spontaneously after any number of subcultures on laboratory media in some strains, and eventually all strains may produce it. One strain (C 407) of a group of five which were plated daily on blood agar suddenly produced this variant

$$\begin{tikzcd}
\text{Bordet-Gengou subculture} & (5) \arrow[r] \arrow[dr] & (6) \arrow[r] \arrow[dr] & (7) \arrow[r] \arrow[dr] & (8) \arrow[dr] & & \\
\text{1st blood agar plate} & & - & + \arrow[dr] & + \arrow[dr] & + \arrow[dr] & \\
\text{2nd blood agar plate} & & & & - & + & +
\end{tikzcd}$$

the other four remaining negative. Viable counts of subcultures 7 and 8 gave a ratio of variant to parent of $1:800 \times 10^6$ on both occasions.

In other respects the strains carrying this variant appeared to be typical Phase I organisms (Table 1); the variant behaved as the parent strain for a few subcultures on blood agar, but on further culture rapidly lost some of its characters (see Table 1). In this respect it is interesting to note that the protective antigen, as measured by the ability to protect mice against an intracerebral challenge, was one of the more stable characters.

Agglutination

One strain was not agglutinable during the first four daily subcultures either by slide agglutination or by tube agglutination, but the 5th and several subsequent subcultures were agglutinated to titre. Occasionally strains were found which did not agglutinate on the slide because of a pronounced pro-zone effect which was obvious in the tube agglutination and could be avoided by diluting the serum used for the slide test, when a positive agglutination was obtained.

Table 2. *Agglutination of* 105 *freshly isolated strains of* Haemophilus pertussis *by several sera prepared against freshly isolated strains*

Titre	No. of strains
16,000–32,000	93
8,000	5
4,000	6
2,000	1

All strains examined agglutinated with Phase I serum, usually but not always to a high titre. The tests on 105 freshly isolated strains in Table 2 show that a small number of strains are of consistently low agglutinability. Other workers have found similar strains (Smolens *et al.* 1947; Kendrick *et al.* 1949; Cohen, 1949). In a further examination five of these strains (Table 3) did not differ appreciably from strains of high agglutinability in any other way.

Table 3. *General characters of five strains of* Haemophilus pertussis *of low agglutinability*

Strain no. and subculture	Growth on blood agar	Agglutination titre	Alum precipitability (%)	Haemagglutinin titre	Protective antigen (ImD 50 × 10^9)	Virulence (intranasa (LD 50 × 1
L 51–7	–	8000	nt	96	0·85	1·0
L 67–7	–	8000	100	12	0·71	nt
W 1276–3	–	4000	100	24	nt	0·1
W 1505–3	–	4000	100	nt	nt	45·0
W 1506–3	–	2000	100	12	0·45	nt

–, negative. nt, not tested. Compare also Table 7, strain P 34–7 and P 54–15.

Antisera were prepared against four freshly isolated strains, two of high agglutinability and two of low agglutinability and subjected to cross-agglutination and cross-absorption tests (Table 4). There was no difference in the major antigens of the two types of strain.

The work of Smolens *et al.* (1947) showed that strains can differ widely in their content of agglutinogen. In general, they found that strains high in

Table 4. *Cross-absorption tests of two strains of* Haemophilus pertussis; *one* (*H*) *of high agglutinability, and the other* (*L*) *of low agglutinability*

Serum			Titres after absorption by			
			Absorbing dose (mg. wet weight)			
Against	Absorbed by	Tested with suspension	0·0	2·5	5·0	10·0
H	H	H	32,000	4,000	50	nt
		L	4,000	4,000	0	nt
	L	H	32,000	nt	2,000	100
		L	4,000	nt	1,000	100
L	H	H	16,000	2,000	0	nt
		L	4,000	1,000	0	nt
	L	H	16,000	nt	100	0
		L	4,000	nt	100	0

nt = not tested.

absorbing power were agglutinated to a high titre, and strains of low absorbing power to a low titre, but there were several exceptions. In my experiments seven strains agglutinating to 1/16,000–1/32,000 had absorbing potencies from 2·5 to 40·0 mg.; a range very similar to the 1·3–10 mg. reported by Smolens. Two strains of low agglutinability were found, one was agglutinated to a titre of 4000 and had an absorption titre of 2·5 mg., and with the other the corresponding figures were 8000 and > 20 mg. Cross-absorption tests with these strains showed that strains of high absorbing power absorb a lot of agglutinin from sera prepared with strains of high or low agglutinability and strains of low absorbing power absorb little from either type of sera. Agglutination, before and after absorption, of heterologous and homologous suspension showed that only one major antigen was involved, so that all these strains must be regarded as agglutinating to titre with Phase I serum. In this respect these strains differed from certain strains of lowered agglutinability obtained by subculture in which another antigen appeared which was demonstrated by cross-absorption tests.

From my experiments and those of Smolens *et al.* we must conclude that the agglutinability of cells and their agglutinogen content vary independently.

Stability of Phase I agglutinogen of strains on continued subculture

Six strains subcultured daily on Bordet-Gengou media were tested at intervals by agglutination and agglutinin absorption. By chance they were all (Table 9) strains of high agglutinability, three of them (L 46, L 49 and L 54) had a high absorbing power (titre 2·5 mg.), one (L 57) a very low absorbing power (titre *c.* 40·0 mg.). All the strains immediately lost absorbing power but not, however, in parallel with the loss of agglutinability, which occurred at different times and at different rates in four of the six strains. Furthermore, apart from the general degeneration of these strains, neither absorbing powers nor agglutinability were lost in parallel with any other character.

One of the strains, L 54, was examined by cross-absorption tests. Subculture 162 differed markedly from the freshly isolated strains of low agglutinability reported above in that another antigen was easily demonstrated which was present neither in subculture 15 nor in any freshly isolated strains of high or low agglutinability. This is presumably the Phase II or Phase III antigen.

Virulence

The virulence (LD 50) for mice by the intranasal route has been dealt with in detail elsewhere (Standfast, 1951). Briefly forty-three strains were tested at the 2nd or 3rd subculture; thirteen of these strains had a LD 50 of less than 1 million, seven between 1 million and 5 million, nine between 5 million and 25 million, thirteen between 25 million and 125 million and one more than 125 million; in fact the LD 50 ranged from 0·1 million to 152 million. These strains grew only on Bordet-Gengou medium and agglutinated with Phase I serum to a high titre.

The eight strains (p. 534) which carried (2nd–4th subculture) the variant growing on blood agar at first isolation were in no way abnormal in virulence.

The virulence of cultures grown on blood agar seems to depend on the number of times they have been subcultured, but varies somewhat from strain to strain (see Table 1), but the loss in virulence is not contemporaneous with the ability to grow on blood agar.

Kendrick *et al.* (1949) examined seventy smooth cultures for virulence by the intracerebral route and found that they fell into two groups, those having an end-point of 10^4–10^7 and those having an end-point of 10^3 or less. The LD 50 of the fifteen strains which fell into the latter group ranged from 100 to 1000 organisms.

I was able to test thirty-one strains for virulence by the intranasal route and the intracerebral route in parallel (Table 5). There was a wide variation in the LD 50 by the intracerebral route ranging from $<10^3$ to 10^9.

Table 5. *Comparison of virulence by intranasal and intracerebral routes of thirty-one freshly isolated strains of* Haemophilus pertussis

(Numbers of strains in each group.)

Intracerebral LD 50	Intranasal LD 50 in millions		
	$<1{\cdot}0$	$1{\cdot}0<10{\cdot}0$	$10{\cdot}0<100{\cdot}0$
$<10^3$	4	1	0
10^4, $<10^5$	0	2	0
10^5, $<10^6$	8	0	1
10^6, $<10^7$	8	2	2
$>10^7$	1	2	0

Virulence by the intercerebral route and virulence by the intranasal route depend on different factors. First, there is no direct correlation between results by the two routes (Table 5); secondly, though the LD 50 by the intranasal route is dependent on the weight and age of the mice (Standfast, 1951) this factor does not influence the LD 50 by the intracerebral route unless very small mice are used.

The intraperitoneal route is more a measure of the toxin content of the organisms than of their virulence. Nine strains were tested in parallel by the intranasal, intracerebral and intraperitoneal route; by the intranasal route the LD 50 varied between $0{\cdot}26\times10^6$ and $15{\cdot}5\times10^6$—a 60-fold range—by the intracerebral route between $0{\cdot}18\times10^6$ and $2{\cdot}5\times10^6$—a 14-fold range—and by the intraperitoneal route between 280×10^6 and 800×10^6—a 2·85-fold range. There was no correlation between high and low value by any of these routes with any particular strain. Table 6 shows the highest and lowest values for each route for the strains tested. The results by the three routes are obviously dependent on different factors and vary independently.

Stability of virulence in strains on continued subculture

On subculture on Bordet-Gengou medium some strains retained their virulence during a large number of subcultures while others lost it quickly. Virulence once lost was not recovered by further subculture, but could sometimes be partly recovered by mouse passage. Table 9 shows some examples of

Table 6. *Comparison of virulence by three routes of five strains of* Haemophilus pertussis

Strain	LD 50 in millions		
	Intranasal	Intracerebral	Intraperitoneal
L 5,743	0·26	0·18	655
L 5,754	15·5	0·24	640
C 10,487	1·0	1·0	800
C 19,767	2·1	1·6	280
C 19,764	3·3	2·5	320

stable and unstable strains. Strains L 54 and L 58 retained their full virulence for 162 and 132 subcultures; the other four strains all lost virulence, but at different times and to a different degree. Strain L 46 has lost virulence most rapidly having an LD 50 of 39 million, at the 39th subculture while strain L 49 maintained its original virulence for at least 145 subcultures and then degenerated. Another strain, C7988, behaved like L 46, losing virulence rapidly, an LD 50 of 4·5 million at subculture 2 fell to 46·0 million by subculture 12. Other strains, such as C2188, behave like L 49, tested on five occasions, up to subculture 206, it had at each test an LD 50 of less than 0·2 million. But at the 218th subculture the LD 50 was 12·0 million and at the 286th subculture, 10·0 million. This drop in virulence followed by stability at the raised figure, as shown by strain L 46 (Table 9) and C2188 but not by L 45, suggests the sudden loss of one of the factors which determines virulence.

Haemagglutination

Seventy-one freshly isolated strains were tested for haemagglutinin on several occasions and gave consistent titres as follows: twenty strains 1/196 to 1/96, twenty-nine strains 1/48 to 1/24, eight strains 1/12 to 1/6, three strains showed traces only at 1/6 or 1/3 and eleven strains were invariably negative. As shown in a previous paper (Standfast, 1951) there is no correlation between haemagglutinin and virulence measured by the intranasal route nor does haemagglutinin tie up with any of the other characters. Table 7 shows the main characters of twelve strains arranged in order of haemagglutinin production. There is no association with any of the other characters (see also Table 9).

Stability of haemagglutinin in strains on continued subculture

Haemagglutinin appears to be one of the more labile characters of *H. pertussis*. Seventeen strains were subcultured daily on Bordet-Gengou medium and tested at various subcultures for haemagglutinin. Table 8 shows four characteristic results.

Protective antigenicity

Single strain vaccines were prepared from eighteen freshly isolated strains and tested for protective potency in mice against a reference vaccine. Most of these vaccines were tested on several occasions and an average ImD 50 obtained. Their ImD 50 varied from $0{\cdot}21 \times 10^9$ to $1{\cdot}32 \times 10^9$. The errors of the test are

Table 7. *A comparison of the characters of twelve strains of* Haemophilus pertussis, *arranged in order of their haemagglutination titres*

Strain and subculture	Haemagglutination titre	Agglutination titre	Alum precipitability (%)	Growth on blood agar	Protective antigen ImD 50 × 10^9	Virulence (intranasal) LD 50 × 10^6	Qu kil fa
P 37–7	96	32,000	100	–	0·48	1·0	
P 34–7	96	8,000	100	–	0·85	9·0	
P 33–22	48	32,000	100	–	0·62	8·3	
P 43–43	48	16,000	100	–	0·37	66·0	
P 37–9	24	32,000	100	nt	1·07	1·7	
P 54–15	24	4,000	100	–	1·32	4·6	
P 53–8	24	32,000	100	+	0·65	3·0	
P 32–22	12	32,000	100	–	0·58	9·4	
P 42–43	12	32,000	100	–	1·12	34·0	
P 31–10	Trace	16,000	100	–	0·34	0·84	
P 26–5	0	32,000	100	–	0·57	0·64	
P 27–5	0	32,000	100	–	1·19	0·67	

The above characters were tested on the same suspension for each strain; after the other tests had been carrie
1/10,000 thiomersalyl was added and a sterile vaccine prepared for the mouse protection tests. Growth on blood
–, negative; +, positive; nt, not tested.

Table 8. *Haemagglutination titres of four typical strains of* Haemophilus pertussis *subcultured daily on to fresh Bordet-Gengou medium*

Strain no.											
L 59	N	4	10	15	25	.	.	.	.	.	.
		24	12	0	0	.	.	.	.	.	.
L 54	N	.	9	15	24	33	39	.	.	.	.
		.	24	24	12	0	0	.	.	.	.
L 48	N	5	.	17	20	29	40	53	69	.	.
		24	.	24	24	12	12	0	0	.	.
L 51	N	2	7	16	23	34	42	52	70	84	95
		96	96	96	48	24	12	12	6	0	0

N, numbers of subculture. Compare also strains in Table 9.

such that it is doubtful if there is any significant difference in the potency of these vaccines. But between two vaccines picked from the extremes of the range there was a suggestion of a real difference in protective properties. In four tests of vaccine C26,573, the ImD 50 was 0·16, 0·21, 0·22 and 0·26, average 0·21, and of vaccine L 57–5, the ImD 50 was 0·96, 1·27, 1·28, 0·2, average 0·93.

There is no correlation between protective properties or any other Phase I character (see Tables 1, 7 and 9), this corroborates the findings of Kendrick and her co-workers who found no marked differences in protective properties of thirty-six smooth strains or any association between protection and relative degree of virulence as estimated by the intracerebral route.

Stability of protective property of strains on continued subculture

Vaccines were prepared at different subcultures from six strains which had been subcultured daily on Bordet-Gengou medium. Table 9 shows the average ImD 50's of the vaccines prepared from the different subcultures. There is a suggestion that the protective potency of some strains decline, though this is marked only in strain L 45. The protective property appears to be lost in different strains at different times and at different rates.

Precipitation by aluminium phosphate

Ungar & Muggleton (1949) examined thirty-four freshly isolated strains, which agglutinated to high titre (1/8000 or more) with phase I serum, for precipitation with aluminium phosphate. Because all these strains precipitated completely they concluded that precipitation with aluminium phosphate provides a quick method for distinguishing virulent Phase I strains from strains which had deteriorated. My experiments are not entirely in agreement; the majority of freshly isolated strains are positive and lose this character when they deteriorate but several freshly isolated strains which were otherwise typical were not completely precipitable, and certain strains which had lost or were losing some of their other characters were still completely precipitable with aluminium

Table 9. *Comparison of six strains tested after varying number of subcultures*

		Subculture no.								
in	Character	**1–9**	**10–25**	**26–50**	**51–75**	**76–100**	**101–125**	**126–150**	**151–200**	**200–225**
5	Agglutination titre	32,000	32,000	32,000	16,000	8,000	.	0	0	.
	Agglutination absorption, mg.	5·0	10·0	.	15·0	.	.	.	>40·0	.
	Alum precipitability, %	100	100	100	.	0	0	.	.	.
	Growth on blood agar	–	–	–	–	–	+	+	+	.
	Haemagglutination titre	48	48	48	12 0	0	.	.	.	.
	Protective antigen, ImD 50	0·41	0·77	1·15	.	.	.	.	3·32	.
	Virulence (intranasal), LD 50	0·64	9·4	34·0	65·0	.	.	.	80·0	.
3	Agglutination titre	16,000	16,000	16,000	16,000	16,000	16,000	.	.	0
	Agglutination absorption, mg.	2·5	3·75	5·0	.	.	20·0	.	.	.
	Alum precipitability, %	100	100	100	.	.	100	.	.	100
	Growth on blood agar	–	–	–	–	–	–	–	–	+
	Haemagglutination titre	48	48	48	12	6		.	.	.
	Protective antigen, ImD 50	0·71	0·98	0·67	.	.	1·19	.	.	.
	Virulence (intranasal), LD 50	0·46	8·3	66·0	.	.	66·0	.		.
	Agglutination titre	16,000	16,000	.	16,000	.	16,000	16,000	16,000	0
	Agglutination absorption, mg.	.	2·5	.	.	.	10·0	.	20·0	.
	Alum precipitability, %	100	100	.	.	100	.	100	100	100
	Growth on blood agar	–	–	–	–	–	–	–	–	–
	Haemagglutination titre	48	24	24	24	6	.	0	0	.
	Protective antigen, ImD 50	0·63	0·63	.	.	.	.	0·87	1·35	.
	Virulence (intranasal), LD 50	0·26	0·64	.	1·6	.	.	3·0	26·0	.
	Agglutination titre	16,000	8,000	8,000	.	.	8,000	8,000	2,000	.
	Alum precipitability, %	100	100	.	.	100	100	100	100	.
	Growth on blood agar	–	–	+	+	+	+	+	+	.
	Haemagglutination titre	24	24 12	0	0	.	.	.	.	.
	Protective antigen, ImD 50	0·71	0·77	.	.	.	.	.	0·78	.
	Virulence (intranasal), LD 50	1·2	2·6	.	.	.	.	.	1·0	.
	Agglutination titre	16,000	16,000	.	16,000	16,000	16,000	.	16,000	8,000
	Agglutination absorption, mg.	*c.* 40·0	.	.	.	.	>40·0	.	>40·0	.
	Alum precipitability, %	100	.	.	100	.	.	.	100	75
	Growth on blood agar	–	–	–	–	–	–	–	+	+
	Haemagglutination titre	24	24	24	24	24	.	.	.	.
	Protective antigen, ImD 50	0·93	.	.	.	.	.	.	1·27	.
	Virulence (intranasal), LD 50	0·13	.	0·53	0·66				3·0	
	Agglutination titre	16,000	16,000	.	16,000	.	16,000	8,000	8,000	.
	Agglutination absorption, mg.	5·0	.	.	10·0	.	20·0	*c.* 40·0	>40·0	.
	Alum precipitability, %	100	.	.	100	.	.	100	100	.
	Growth on blood agar	–	–	–	–	–	–	–	–	.
	Haemagglutination titre	24	24	.	24	.	0	.	.	.
	Protective antigen, ImD 50	1·13	.	.	.	.	.	1·85	0·95	.
	Virulence (intranasal), LD 50	9·3	.	.	.	.	.	10·0	10·0	.

phosphate (Table 3, Table 9, strains L 45, L 46, L 49; see also Ungar & Muggleton (1949), Table 3, strain 10,203).

In my experiments thirty-nine strains were tested at first isolation; thirty-five precipitated completely, three to about 75 % and one not at all.

Stability of aluminium phosphate precipitability in strains on continued subculture

Of forty-six strains, which had been subcultured in the laboratory on Bordet-Gengou medium up to 100 daily subcultures and were still in Phase I by the other criteria, tested with aluminium phosphate forty-one were 100 % positive, three were about 75 % and two were negative.

Six strains subcultured daily on Bordet-Gengou medium for 225 days were tested at various intervals. In one of these strains there is a complete loss of precipitability from the 48th subculture, in another some loss by the 68th subculture, the other four strains were stable up to the last subculture tested (Table 9). Four variants growing on blood agar were tested after 9, 12, 13 and 13 subcultures on blood agar. These variants were no longer agglutinated to titre with Phase I serum but two were completely precipitated and two precipitated to about 75 % (see also Table 1, strain C 5467).

DISCUSSION

When freshly isolated *H. pertussis* strains are examined to determine the main characters by which so-called Phase I organisms are distinguished from the other phases, three facts come to light. (1) Any one character varies quantitatively and it is therefore impossible, except by setting up an artificial boundary, to use any character qualitatively for definition. One cannot simply say that a certain strain is 'virulent' or 'agglutinates'. (2) Not all strains have all the characters which one normally expects in freshly isolated strain of *H. pertussis*. For example, the strain M 7 in most characters was a typical *H. pertussis* strain, but from first isolation it could grow on blood agar. Portwood (1946), who investigated the catalase activity of *H. pertussis*, reported that fourteen of sixty-two smooth strains had no activity. (3) The main characters vary independently, and the presence of one character even strongly marked is no evidence that another character is also strongly marked or in fact present at all. In addition to evidence presented in the paper, Portwood (1946) reported that agglutination tests presented no evidence of differences in antigenic structure between strains with or without catalase activity.

On subculture, characters may degenerate or be lost in different strains at different times and in different orders. Diagrammatically we may view a strain of *H. pertussis*, as in Fig. 1, where the characters present in the freshly isolated strain degenerate at different rates. Fig. 2 shows a single character in a number of different strains, present in different potencies at first isolation and on culture in the laboratory degenerating at different rates and at different times.

What then is a Phase I organism? No exact definition is possible since this definition must depend to a great extent on opinion. Phase I was originally defined serologically. But Smolens *et al.* (1947), Kendrick *et al.* (1949) and Cohen

(1949) have already reported variations in the agglutinability of freshly isolated strains, and also variations in the absorbing potencies of different strains which did not go hand in hand with their agglutinability (Smolens *et al.* 1947). The serological experiments reported in this paper support the view that freshly isolated *H. pertussis* strains are serologically homologous. They also show that both the agglutinogen content of the cells as measured by agglutinin absorption and the agglutinability of the cells as measured by the agglutination test vary, and vary independently. The significance of the consistently low agglutination titres given by some strains is not clear. By agglutination titre and agglutinin absorbing power freshly isolated strains may be divided into four groups: strains of high agglutinability and high absorbing power, high agglutinability and low absorbing power, low agglutinability and high absorbing power and low agglutinability and low absorbing power; most strains fall into the first group; strains of low agglutinability are relatively rare probably comprising not more than 10 % of strains isolated. Two of the three strains I examined were of high absorbing power and one of low.

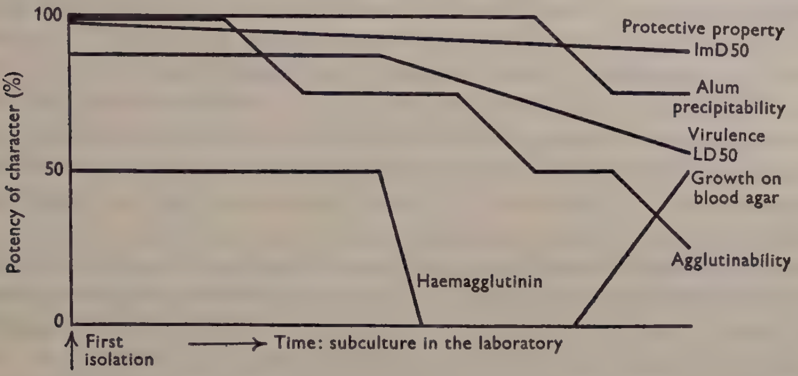

Fig. 1. Schematic representation of characters of a typical strain of *H. pertussis*.

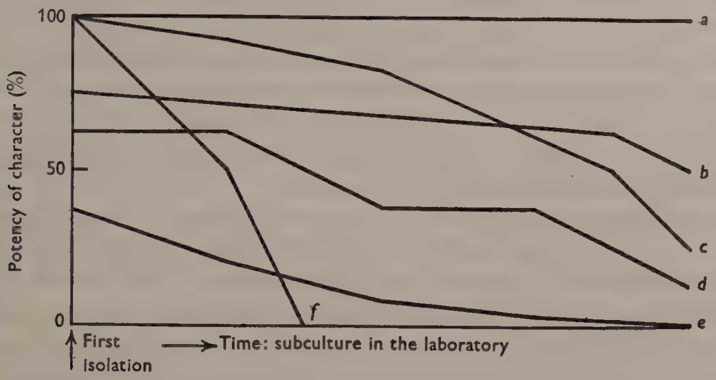

Fig. 2. Schematic representation of the same character in six strains of *H. pertussis*.

It is obvious from cross-agglutination (Table 4) that low agglutinability is a function of the suspension and not of the antiserum, and that only one antigen is involved. Until the strain of low agglutinability and low absorption was found, I thought that strains of low agglutinability were strains with *a lot of agglutinogen* which for some reason required a high concentration of serum to agglutinate them. This, however, is not always so, and furthermore it is unlikely that strains which had been kept in the laboratory and were of lowered agglutinability, would increase their agglutinogen content. It is, however, difficult to understand why two strains with the same agglutinogen content as measured by agglutinin absorption should behave so differently in the presence of the same dilutions of agglutinating serum unless the variation in agglutinability is determined by factors other than the amount of agglutinogen on the cell surface, e.g. the distribution or availability of the receptors on the cell surface. Thus, if the active groups were clustered in discrete patches relatively distant from each other, more serum might be required to agglutinate them than if they were more evenly scattered. This hypothesis is supported, I think, by the behaviour of the old laboratory strains (Table 9). These strains all lost agglutinogen as measured by absorption before they lost agglutinability, which may mean that they first lost active groups and then these groups become 'thinned out' too far. The mechanism of agglutination in this group, of course, may be complicated by the appearance of another major antigen. The behaviour of the two strains of low agglutinability and high absorption may be due to 'bad siting', resulting in lack of availability of the active groups.

These experiments, at any rate, indicate that the serology of Phase I is more complicated than originally thought and the expression 'agglutinates to titre' is not a valid criterion of a Phase I organism.

The word 'phase' with respect to Phase I *H. pertussis*, with its implication of phase as used in the salmonellas, has proved with time to be an unfortunate choice as a synonym for smooth or freshly isolated; it has served its useful purpose and should now be dropped. Leslie & Gardner's original contention that the phases of *H. pertussis* could revert has not, so far as I am aware, been confirmed.

'Freshly isolated' as a defining phrase is also open to objection, as some strains maintain their 'freshly isolated' characters for some time in the laboratory while others lose all or some rapidly.

The term 'virulent', usually meaning virulent for mice, is also open to objection, for though no avirulent, but otherwise typical strain, has yet been isolated, virulence is a quantitative term and the definition of *avirulent* must depend to some extent on dosage.

We fall back therefore on '*Smooth*' as the best description of these strains; this was first suggested by Leslie & Gardner (1931) and stressed by Shibley & Hoelscher (1934) and by Lawson (1939), who pointed out that the dissociation of *H. pertussis* was an example of the S → R variation with one or more intermediate stages, the exact characters of which will depend on the state the organism has reached.

The exact definition of the *Smooth* state with our present knowledge may be no easier, for we are dealing with an extremely variable organism, but we are employing a word in common bacteriological usage with a recognized meaning.

Freshly isolated strains of *H. pertussis* accordingly fall into three groups, the 'ideal' or 'perfect' strains (which like other ideals may not exist in fact) containing all the characters in a fully developed state, 'normal' strains containing all the characters only some fully developed, but all adequately represented; and a third group in which one or possibly more characters are missing or are feebly displayed. All these strains will be *Smooth*.

With a highly developed parasite like *H. pertussis* the final definition of a 'freshly isolated', 'virulent', 'smooth', 'Phase I' strain will probably depend on 'virulence for human beings'. When the factors responsible for this are known a final definition may be possible.

The author is indebted to the Directors of the Public Health Laboratory Service Laboratories at Cardiff, Colindale, Leeds, Manchester, Oxford and Poole for the supply of *H. pertussis* strains; and to Miss M. P. D. Pile and Miss Betty Tredwell for technical assistance.

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Influenza Haemagglutination

An Evaluation of the Photoelectric Titration Method

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SUMMARY: A modification of the Hirst & Pickels photoelectric densitometer and its application to work on influenza virus haemagglutination are described. The methods used are believed to reduce to a minimum the inherent margin of error of virus and serum antibody assays based upon the haemagglutination reaction. In order to make full use of data thus obtained precise assessment of the significance of differences of titre is essential, and for this purpose two simple statistical tests have been applied and their application illustrated by an example.

Several different methods, based upon the haemagglutination reaction, are available for titrating influenza viruses and serum inhibitory substances. Only those, however, which minimize the subjective element in the assessment of agglutination end-points are suitable for certain types of investigation, notably investigations concerned with relatively small differences between samples. The method originally used in this laboratory was a modification of that devised by Hirst & Pickels (1942) in which a photoelectric densitometer is employed to measure the light transmission through each tube of a titration series, and the 50 % agglutination end-point is calculated from the galvanometer readings (Smith & Westwood, 1949). During the past two years several modifications have been introduced and a new photoelectric densitometer constructed with the object of reducing the inherent margin of error of the test to a minimum. The employment of such a method is, however, only justified if full use is made of the data obtained. This requires a precise assessment of the significance of deviations of titre. It is the purpose of this paper to describe the method now in use, how the error of the test has been determined; and to outline two statistical tests for assessing the significance of titre differences.

Technique of the test

A twin-cell instrument of the type used by Needham for recording bacterial growth curves was used (Needham, 1947). The general layout is shown in Fig. 1. The light from a 6 V., 24 W. bulb is allowed to fall simultaneously on two matched photo-cells. The cells are connected in opposition and bridged by a sensitive, centre-reading galvanometer. So long as the same light intensity is falling on both cells, the system remains balanced. When a tube of fowl erythrocyte suspension is placed in front of one cell, the decrease of the light intensity falling on that cell causes a potential difference to develop across the galvanometer terminals, which is registered by a deflexion of the needle. This deflexion is brought back to zero by increasing a potentiometer resistance inserted in the circuit of the opposing cell. The potentiometer reading is thus an accurate measure of the density of the red-cell suspension.

As fluctuations in the intensity of the light source affect both photo-cells simultaneously, the instrument is self-compensating. The 'base line' reading of the instrument is checked by measuring the density of a standard red screen.

The sensitivity of such an instrument is determined by the intensity of the light source, the potentiometer resistance and the sensitivity of the galvanometer. For our instrument the specifications were as follows:

Galvanometer. Weston centre-reading, d.c. type, sensitivity 2 μa. per division.

Potentiometer. Enclosed pattern, 1 K. resistance, 15 W.

Lamp. Car headlight bulb, 6 V., 24 W.

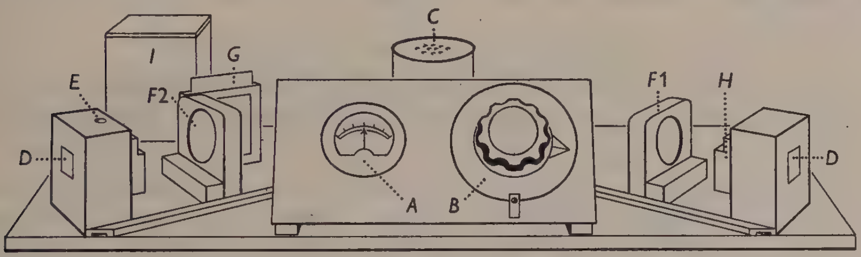

Fig. 1. *A*, galvanometer; *B*, potentiometer dial; *C*, lamp housing; *D*, photoelectric cells; *E*, tube holder; *F*1, 2, lens holders; *G*, standardizing screen (hinged to lens holder, *F*2); *H*, holder for infra-red absorbent screen and balancing filters; *I*, 6 V. transformer.

Hard wood blocks containing holes drilled vertically to a depth of 1½ in. were substituted for the more conventional racks. With tubes inserted the clearance is only just sufficient for smooth manipulation. Thus the tubes containing the test mixtures are held in a strictly vertical position so that maximum sedimentation of the agglutinated cells to the bottom is ensured. This avoids spuriously high density readings due to the accumulation of cell clumps on the sides of the tubes, an error which is common when racks of the ordinary type are used. Glass tubes, 7·0 × 0·8 cm., are carefully selected for internal diameter by checking the column height of a 2 ml. volume of water to within ± 1 mm. of a standard. They are then standardized by filling with a suspension of mammalian red blood corpuscles, and taking density readings; all tubes giving a reading deviating more than ± 1 potentiometer division from a standard are rejected. Mammalian cells are used because of their greater stability in suspension, and to obviate the streaming effect which occurs in fowl cell suspensions.

The test must be precisely timed. At the end of 75 min. standing, sedimentation is still proceeding rapidly in those tubes showing agglutination, and the 50 % end-point is moving towards the higher dilution region. A delay in reading of 1 or 2 min. may produce a considerably enhanced titre.

Where the 50 % agglutination end-point falls between two dilutions it is calculated by logarithmic interpolation as in Hirst's original method (Hirst & Pickels, 1942).

The error of the test

Since the titre is calculated by logarithmic interpolation it may theoretically attain any value between consecutive dilutions, and may thus be regarded as a continuous variable. The standard error of a titre may be estimated by replicating the titration, but it must be noted that the actual observations obtained may be biased by the operator performing the test. The original experiments carried out to measure the standard deviation of both virus and antibody titrations were so designed that the operators' bias could be extracted from the data by analysis of variance. This technique had the further advantage that it was possible to obtain additional information from the experiment, e.g. whether there was a significant difference in the titres between operators, and whether alternative methods of making the twofold dilutions produced different results.

Table 1. *Replicate titrations by three different operators*

Titres of a virus suspension estimated in replicate tubes by three operators

Row	M. A. W.		G. B.		J. C. W.	
	Single pipette	Multiple pipette	Single pipette	Multiple pipette	Single pipette	Multiple pipette
1	290	282	285	310	269	261
2	277	255	209	286	331	255
3	282	233	252	282	266	249
4	243	251	265	320	289	255

Table 2. *Partial analysis of variance of titres in Table* 1

Source of variation	Sum of squares	D.F.	Mean square	Variance ratio	*P*
Operators	592·40	2	296·20	—	—
Methods	15·08	1	15·08	—	—
Operator-Methods	7264·27	2	3632·14	6·95	<0·01>0·001
Residual Error	9403·00	18	522·39	—	—

Each of three operators carried out eight titrations on the same virus preparation, four in which a single pipette was used for each dilution series and four in which a fresh pipette was used for each dilution. The same fowl cells were used throughout. The titres obtained in this experiment are tabulated in Table 1, and the analysis of variance is shown in Table 2. The analysis is incomplete, because the errors due to the operators and operator-methods are not separated, but in the present instance the information obtained by separating these errors is irrelevant. It is clear that there is no significant difference between operators or methods of dilution *per se*, but the operators differed in their use of the dilution methods, and the difference is highly significant. The standard deviation of the whole sample is the square root of the error mean square.

This type of experiment has been repeated a number of times, and the results compared with the standard deviations of samples from individual operators. The coefficient of variation for the virus titrations varied between 5·5 and 9·5 % with a mean of 7·8 %, and for the serum antibody titrations between 5·5 and 9 % with a mean of 6·6 %.

Significance of differences between titres

There are two methods of assessing significant differences. An arbitrary limit may be adopted or the difference may be assessed against the error of the experiment. The assignment of arbitrary limits is unsatisfactory; they may either be set so finely relative to the error of the test as to lead to dangerous conclusions, or so widely as to be wasteful of information. It is clearly of little value to perfect an exacting technique, merely to subject the data it provides, to wide arbitrary limits of significance. Nevertheless, this method is that most widely used in haemagglutination titration work.

Tests of significance between single observations

The determination of the significance of the difference between the titres of two samples is not difficult. The comparison may be made as precise as is necessary, merely by replicating the titrations of each sample a sufficient number of times and comparing the means of such replications, using 'Student's' *t* test. The error in such a case is directly estimated from the data. This method is statistically ideal, and it must be clear that it requires no previous standardization of the test for error.

It is not, however, possible in haemagglutination experiments always to carry out an adequate number of replications, and the question frequently arises as to whether two single observations are or are not significantly different. Data consisting of two single measurements cannot supply their own estimate of error. This must, therefore, be pre-determined. If the variable were normally distributed and its standard error, σ, were constant for all its values, then we could use the test criterion

$$\frac{x_1-x_2}{\sqrt{(2S)}}=t,$$

where x_1 and x_2 are the two single observations and S is the previously determined estimate of σ.

But in haemagglutination titrations the standard error (σ) of the titre (x) is not constant for all values of x, although it appears that the ratio of the standard deviation to the mean (coefficient variation) does remain constant. Denoting the coefficient of variation by Vc we have $Vc=\sigma/x=$ constant and we can, therefore, obtain an estimate of σ for any particular value of x from a previously determined estimate of Vc. It thus appears that the test criterion in this case should be

$$\frac{x_1-x_2}{Vc\bar{x}}=M_t, \qquad (1)$$

where $\bar{x}=\frac{1}{2}(x_1+x_2)$. In order to calculate significance points for this expression it is necessary to consider the distribution of M_t which depends on the distribution of x. In haemagglutination titrations it would appear that the titre, x, is not in fact normally distributed, but $\log x$ is so distributed. On this assumption, and assuming that the correct value of Vc is used, it can be shown that the 1% significance point of M_t is approximately 3·6 and the 5% point is 2·8. It is interesting to note that these significance points are almost constant for true values of Vc between 4 and 10 %. It is obvious, however, that if the previously determined value of Vc were larger than the true value, then the ratio $(x_1-x_2)/Vc\bar{x}$ would always be too small and significant differences between x_1 and x_2 would be missed. Also if the estimate of Vc were too small, then the test would give too large a number of significant differences. Since the estimates of Vc varied between 5·5 and 9·5 % it was decided, before the significance points had been fully examined, to take the value 10 % thus ensuring that differences were not wrongly considered significant, and the 1 % significance point of M_t was taken as 3. It appears, however, that the use of this smaller value was compensated for by the use of the overestimate of 10 % for Vc. It was found that if the true value of Vc were 8·3 % and the value used in calculating $(x_1-x_2)/Vc\bar{x}$ was the overestimate of 10 % then the 1 % significance point of M_t would be reduced to 3. From the data the average estimate of Vc was 7·8 %, which suggests that 3 was a safe value.

The logarithmic transformation. It requires such a very large number of observations to obtain the exact distribution of a variable that a direct test was impracticable in this case. However, tests of significance are less affected by slight deviations from normality than by changes in the standard error. Though the standard error of x increases as x increases, the standard error of $\log x$ remains constant. It was, therefore, assumed that $y=\log x$ was normally distributed, and this assumption was supported by the fact that the titre was calculated by interpolation of values of $\log x$. It was on this assumption that significance values of M_t were obtained.

However, it seems more convenient to use the normal variable y in testing for differences, since tables of the normal curve and 'Student's' t distribution have been widely published. The significance test of two individual values of x is made in the following way. An estimate of the constant standard error of y (σ_y) is made from previous experiments and it is seen that

$$\frac{y_1-y_2}{\sqrt{(2\sigma_y)}}=t, \tag{2}$$

where $y_1=\log x_1$, $y_2=\log x_2$ and the number of degrees of freedom are those on which the estimate of σ_y is based. If the number of degrees of freedom is large, then tables of the normal distribution may be used.

It is one of the advantages of this method that a value of P for any difference can be measured.

An example of the tests of significance

In a cross-immunizing experiment, two sera, A and B, obtained in two successive bleedings from the same animal, were titrated for influenza-virus antibody by the haemagglutination inhibition titration. The titres (x_1 and x_2) were 639 and 755 respectively. The tests of significance, to determine whether the difference between the two titres represents a true antibody rise, or merely represents random error, are applied as follows.

The 'M_t' test. *Vc*, previously determined for haemagglutination inhibition titrations, $=6{\cdot}6\ \% = 6{\cdot}6/100 = 0{\cdot}066$; and $\bar{x} = (639+755)/2$. Substituting these values in equation (1), we have

$$M_t = \frac{755-639}{0{\cdot}066 \times \frac{1}{2}(639+755)} = 2{\cdot}5.$$

The 5 % probability value for M_t is 2·8. A deviation between two antibody titres as great as that observed, would be obtained as a result of random error more than once in twenty trials. It is unlikely, therefore, that the difference is significant.

The logarithmic transformation. Applying equation (2), we have, $\sigma_y = 0{\cdot}061$, determined experimentally from a series of titrations and, being the standard error of log x, calculated in the usual way. Then

$$t = \frac{2{\cdot}8779 - 2{\cdot}8055}{0{\cdot}061} = 1{\cdot}187.$$

Since σ_y was determined with 88 degrees of freedom, the value of P corresponding to $t = 1{\cdot}187$ is $0{\cdot}3 > P > 0{\cdot}2$. The difference in titres observed will occur as a result of random error with a mean frequency of between two and three times in every ten trials. It is, therefore, unlikely to be a significant deviation. It is worth noting that the logarithmic transformation gives a more precise estimate of P than the M_t test.

DISCUSSION

The method described is of general applicability and can be used to measure the significance of differences between single observations in any test of which the parameter σ_y^2 can be reliably estimated and y is normally distributed. It is not, however, applicable to techniques where the titre is restricted to one of a series of tube dilutions or to an arbitrarily interpolated value, since in such cases the titre does not form a continuous variable. It is essentially a research method. For routine work the significance of small differences of titres are not likely to be important. Significance in the diagnostic sense is not concerned with the question of whether two titres differ statistically, but whether the difference justifies a positive diagnosis. It has been shown, for instance (Wolff, 1949), that a fourfold increase in antibody level is required to justify such diagnosis in the case of influenza. Therefore, for work of this nature, a simpler method such as the Salk test is preferable, possessing as it does the great advantage that it does not require any special apparatus (Salk, 1944).

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Zinc in the Metabolism of a Strain of *Aspergillus niger*

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SUMMARY: Cultures of *Aspergillus niger* partially deficient in zinc produce much larger quantities of acid per unit of glucose used than do normal cultures. This fact has been the basis of the current suggestion that zinc plays a part in the utilization of carbohydrate by fungi. The experiments described here show that partial deficiencies, not only of zinc but also of iron, copper, manganese, phosphorus, magnesium and potassium all had a similar effect on the production of acid by a strain of *A. niger*. It would appear that the effect of zinc on the way in which carbohydrate is utilized is not a specific effect and indicates no more about the role of zinc than about the role of any other inorganic nutrient.

It has been suggested (Foster, 1939; Foster & Waksman, 1939; Foster, 1949) that zinc plays a part in the utilization of carbohydrate by fungi because under conditions of partial zinc deficiency less carbohydrate is oxidized completely and a greater proportion is diverted to form organic acids (Butkewitsch & Orlow, 1922; Bortels, 1927; Waksman & Foster, 1938; Foster, Woodruff & McDaniel, 1943; Foster & Waksman, 1939; Shu & Johnson, 1948). Foster & Waksman (1939) concluded that zinc has a specific effect on carbohydrate metabolism.

Experiments on the metabolism of *A. niger* when partially deficient in several other inorganic nutrients as well as zinc are described in this paper. Certain of these findings have already been reported briefly elsewhere (Chesters & Rolinson, 1950).

METHODS

Surface cultures of a strain of *A. niger* isolated in this laboratory were grown in 100 ml. wide-mouthed conical Hysil or Pyrex flasks. The flasks were cleaned with chromic-sulphuric acid mixture, washed six times with tap water, twice with distilled water and once with double-distilled water prepared in Pyrex glass stills. Each flask was closed with a plug of non-absorbent cotton-wool wrapped in muslin. The flasks were sterilized in the autoclave at 15 lb./sq.in. for 15 min. and 20 ml. of medium used per flask. The basal culture medium contained (w/v): glucose, 5%; NH_4NO_3, 0·3%; $MgSO_4.7H_2O$, 0·05%; KH_2PO_4, 0·05 %; Fe, 0·5 p.p.m.; Zn, 0·5 p.p.m.; Cu, 0·05 p.p.m. and Mn, 0·05 p.p.m. This medium was freed from traces of zinc, iron, copper and manganese by shaking 750 ml. with two successive quantities of approximately 0·1 g. of 8-hydroxyquinoline (oxine) and 5 ml. chloroform in a 1 l. Pyrex separating funnel at pH 7·4 and then again at pH 5·2. Nitric acid (Analar) was used to adjust the pH. After each extraction with oxine the solution was washed twice with 5 ml. chloroform. The medium was finally washed three times with 5 ml. chloroform and once with 10 ml. to remove all traces of oxine. B.P. grade chloroform was found to be satisfactory. Any chloroform remaining in the medium was removed during autoclaving. After purification, each trace element was then added in the quantity required, using stock solutions prepared from A.R. grade reagents and double-distilled water.

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Inoculation was carried out by transferring a mass of spores from a 4-day culture and distributing them on the surface of the medium with a sterile platinum wire. The cultures were incubated at 28°.

Mycelial weights were determined by filtering the pellicles on tared filter-papers in a Büchner funnel and weighing the papers together with the mycelium after drying overnight at 80°.

RESULTS

The effect of zinc deficiency on the production of acid

Cultures containing graded amounts of zinc were harvested at 4 days and analysed. The results are shown in Fig. 1. Maximum acid accumulation occurred when zinc was partially deficient. Growth at this point was approximately 70 % of the maximum. With larger quantities of zinc, less acid accumulated and more growth was made for the amount of glucose used. Fig. 2 shows the result of analyses made at different times during the fermentation by partially deficient and normal cultures. These results show that even the normal cultures do produce a considerable amount of acid but that at 4 days it has been almost entirely metabolized by the fungus. Acids are produced until the glucose becomes exhausted, whereupon the acids themselves are utilized as carbon sources. At 4 days, most of the acid in the normal cultures has been utilized, whereas in the partially deficient cultures the accumulated acid is almost entirely intact. In this respect, analyses at 4 days give an exaggerated account of the effect of zinc on the formation of acid. Nevertheless, more acid is produced by cultures partially deficient in zinc than by normal cultures.

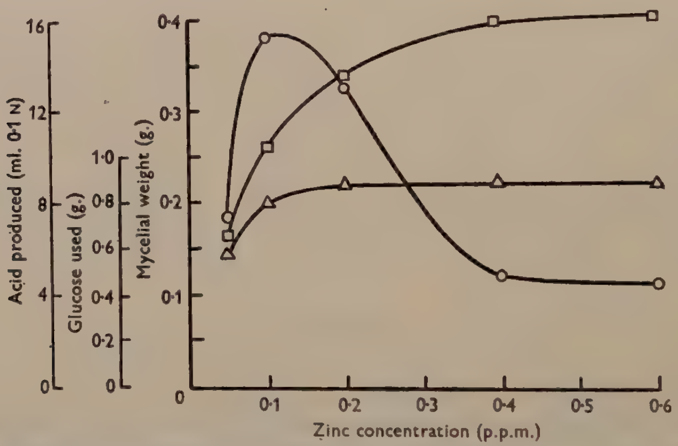

Fig. 1. The effect of zinc deficiency on the growth (□—□), glucose uptake (△—△) and acid production (○—○) of *A. niger* after 4 days' incubation.

The effect of deficiencies of certain other inorganic nutrients on acid metabolism

Cultures containing graded amounts of iron, copper, manganese, magnesium, potassium or phosphorus were harvested at 4 days and analysed. The results are shown in Tables 1 and 2. It will be seen that partial deficiencies of iron, copper, manganese, magnesium, potassium or phosphorus have the same effect on the production of acid as a partial deficiency of zinc. Certain of these cultures were analysed after *c.* 48 hr. when the accumulation of acid in the normal cultures was at a maximum. As shown above, a more accurate account of the formation of acid is given at this time. These results are shown in Table 3. It will again be seen that for each metal, more acid is produced per unit of glucose used when the culture is partially deficient.

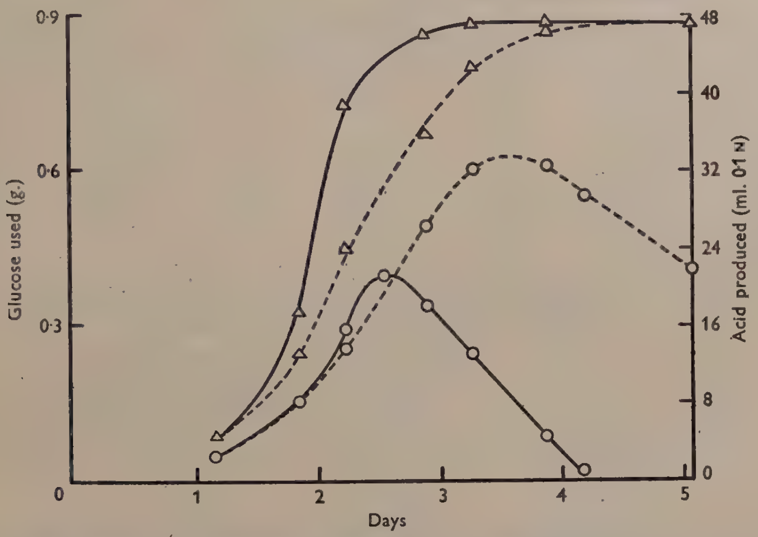

Fig. 2. The effect of zinc deficiency on the course of metabolism of *A. niger*. Full lines, 1·0 p.p.m. of zinc present in the medium. Broken lines, 0·15 p.p.m. of zinc present in the medium. △, glucose uptake; ○, acid production.

Table 1. *The effect of zinc, iron, copper and manganese on acid production by a strain of* Aspergillus niger

Metal in medium (p.p.m.)	Zinc		Iron		Copper		Manganese	
	Mycelial wt. (g.)	ml. 0·1 N acid	Mycelial wt. (g.)	ml. 0·1 N acid	Mycelial wt. (g.)	ml. 0·1 N acid	Mycelial wt. (g.)	ml. 0·1 N acid
0	—	—	—	—	0·250	7·5	0·29	33·5
0·005	—	—	—	—	0·310	33·0	0·359	18·3
0·01	—	—	0·135	15	0·322	24·7	0·348	17·0
0·02	—	—	0·197	30	—	—	0·374	15·2
0·05	0·150	10·1	0·264	49·6	0·362	8·2	0·372	13·4
0·1	0·230	29·9	0·328	43·7	—	—	—	—
0·2	0·351	16·0	—	—	—	—	—	—
0·5	0·382	5·0	0·367	13·0	0·339	4·0	—	—

Table 2. *The effect of phosphorus, magnesium and potassium on acid production by* Aspergillus niger

(When the concentration of potassium dihydrogen phosphate was varied the concentration of potassium was held constant by the addition of potassium chloride. In the case of potassium chloride the potassium dihydrogen phosphate in the medium was replaced by sodium dihydrogen phosphate.)

Concentration (g./100 ml.)	Potassium dihydrogen phosphate		Magnesium sulphate		Potassium chloride	
	Mycelial wt. (g.)	ml. 0·1 N acid	Mycelial wt. (g.)	ml. 0·1 N acid	Mycelial wt. (g.)	ml. 0·1 N acid
0·002	—	—	—	—	0·112	6·6
0·005	0·100	—	0·172	7·7	0·219	9·7
0·01	0·167	6·9	0·312	20·6	0·318	25·1
0·02	0·326	33·3	0·336	4·9	0·339	6·4
0·03	0·351	18·0	0·344	3·0	0·337	1·8
0·05	0·390	4·6	—	—	—	—

Table 3. *The effect of deficiencies of iron, copper and manganese on acid production after* 50 *hr. incubation*

Nutrient	Concentration (p.p.m.)	ml. 0·1 N acid produced/g. glucose used after 50 hr. incubation
Iron	0·05	34
	0·5	17·2
Copper	0·01	37·8
	0·5	25·4
Manganese	0·0	35·0
	0·05	27·8

The results of Tables 1–3 show that although a deficiency of zinc results in the formation of larger quantities of acid, this effect is by no means peculiar to zinc. Partial deficiencies of all the inorganic nutrients examined have the same effect. It is not clear how a deficiency of zinc or any other inorganic nutrient affects the manner in which glucose is metabolized.

The effect of zinc on the amount of glucose used

Cultures were grown in a medium containing 0·15 p.p.m. of zinc; at this concentration, growth was 75 % normal. After 3 days' growth, the medium was removed aseptically and replaced with fresh sterile medium. In one set of flasks this medium contained 10 p.p.m. of zinc, in a second set 0·5 p.p.m. and in a third set, no zinc. The NH_4NO_3 was omitted from the replacement medium so as to diminish subsequent growth of the mycelial felts. The cultures were analysed at intervals and the results are shown in Table 4.

Table 4. *The effect of zinc on the uptake of glucose*

Concentration of zinc (p.p.m.)	Glucose used (g.)			Mycelial wt. (g.)	Acid produced (ml. 0·1 N)	
	24 hr.	48 hr.	72 hr.	72 hr.	48 hr.	72 hr.
0	0·27	0·41	0·49	0·38	30·3	36·1
0·5	0·27	0·45	0·46	0·38	24·1	28·2
10·0	0·28	0·40	0·47	0·39	13·7	17·6

Although the presence of zinc altered the manner in which glucose was utilized, the metal had no significant effect on the amount of glucose used.

The effect of zinc on the utilization of the acids formed during the fermentation

Cultures containing 0·15, 0·2 and 2·0 p.p.m. of zinc in the medium were incubated, and the utilization of the acids formed during the early part of the fermentation was followed. The results are shown in Fig. 3. Although cultures partially deficient in zinc produce larger quantities of acid, such cultures are capable of utilizing the accumulated acids at the same rate as those cultures supplied with adequate amounts of zinc.

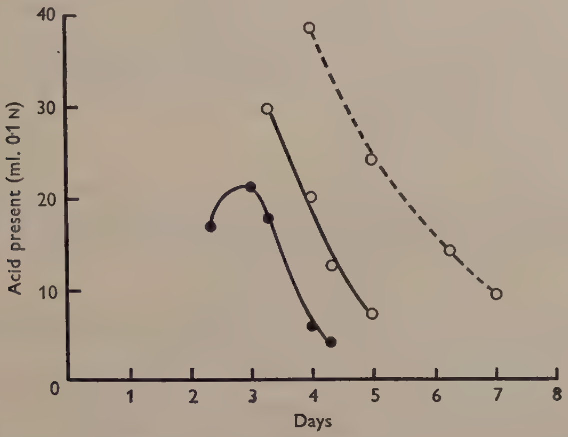

Fig. 3. The effect of zinc deficiency on the utilization of acids formed during growth. The concentrations of zinc in the media were 2·0 p.p.m. (●——●), 0·2 p.p.m. (○——○) and 0·14 p.p.m. (○---○).

DISCUSSION

A deficiency of zinc undoubtedly affects the way in which glucose is metabolized. When zinc is partially deficient, more glucose is diverted to form organic acids and less is oxidized completely to carbon dioxide and water. From the point of view of liberating energy, this acid fermentation is a wasteful process and less growth is possible per unit of glucose used. Zinc therefore affects the 'economic coefficient' (mycelial weight/glucose used) of the organism. Javillier (1912*a*, *b*) referred to this as a physiological characteristic of the metal, and Foster (1939) and Foster & Waksman (1939) concluded likewise that zinc has a specific effect on carbohydrate metabolism. However, the results presented here show that iron, copper, manganese, magnesium, potassium and phosphorus as well as zinc, all have the same effect on acid production and on the efficiency of glucose utilization at least by this strain of *A. niger* and that these effects cannot be regarded as characteristic of a deficiency of zinc alone.

The conception that zinc has a specific effect on carbohydrate metabolism may be due to the relative ease with which zinc deficiencies in fungi can be obtained. Culture media may be partially deficient in this metal without any special attempt to make them so. Iron, and especially copper and manganese on the other hand are usually present as impurities even in A.R. grade reagents in sufficient quantities for maximum growth, and the addition of these nutrients to such a medium would obviously have no effect.

It would appear from the results presented here that rather less is known of the precise role of zinc than the published work would seem to suggest. From all the work with fungi on the effect of zinc on the economic coefficient and on the accumulation of various organic acids, no more can be concluded about the role of zinc than about the role of any other inorganic nutrient. Experiments on the effect of zinc on carbohydrate metabolism show that the metal has no effect on the rate of glucose uptake nor on the rate at which the accumulated acids are subsequently metabolized.

The authors wish to express their gratitude to Boots Pure Drug Co. Ltd., for the award of a Fellowship to one of us (G. N. R.) to enable this work to be carried out.

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Trace Elements and Streptomycin Production

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SUMMARY: Using a defined medium capable of purification free from trace elements the relationship between the latter and streptomycin production by *Streptomyces griseus* was investigated. Zinc and copper were essential for antibiotic production in as much as they were essential for growth. In the case of iron, approximately five times as much was required for antibiotic production as for growth alone. It was not possible to demonstrate that manganese was essential for either growth or antibiotic production.

The experiments reported below were carried out to investigate the relationship between trace elements and antibiotic production by *S. griseus* and to find whether those trace elements already shown to be indispensable for the growth of higher plants, algae, fungi and bacteria are also required by this actinomycete. As far as we are aware, such trace elements have not been shown to be essential for any species in the Actinomycetales. Some of the present findings have already been reported briefly elsewhere (Chesters & Rolinson, 1950).

Certain statements have been made concerning the growth and antibiotic production of *S. griseus* in relation to trace elements. Woodruff (1947) found that zinc favoured growth but inhibited streptomycin production, and conversely, iron depressed growth but enhanced streptomycin production; the optimum concentration of iron was found to be 30 p.p.m. and of zinc, 10 p.p.m. Spilsbury (1948) reported that the addition of 50 p.p.m. of iron had no significant effect on either growth or streptomycin production, and that 10 p.p.m. of zinc decreased both growth and production of streptomycin. Johnstone & Waksman (1948), using *S. bikiniensis* found that, whereas iron had little effect on antibiotic production, 50 p.p.m. zinc sulphate had a considerable stimulating effect, and, when both ions were added, iron partly neutralized the favourable effect of zinc. Both iron and magnesium were reported by LePage & Campbell (1946) to give a general increase in the yield of streptomycin. According to Reynolds & Waksman (1948) iron has a very favourable effect on the production of grisein by *S. griseus*. It is evident that trace elements influence the growth and antibiotic production of *S. griseus* but the precise relationships are confused.

METHODS

Care was taken throughout to ensure that the growth medium and glassware were rendered as free as possible from the trace elements under investigation, and that subsequent contamination by these elements was avoided. All water used was distilled twice in a Pyrex-glass still. The defined medium described by Lumb (1949) was used throughout the experiments. Traces of zinc and copper were removed by extraction with chloroform solutions of diphenylthiocarbazone (dithizone) at pH 7·3 (Feeney, Lightbody & Garibaldi, 1947). Iron and manganese were removed in a similar manner, using 8-hydroxyquinoline (oxine) (Waring & Werkman, 1943) at pH 5·2 for iron and pH 7·3 for manganese. In all experiments the concentration of one trace element was varied in turn while the remaining trace elements were kept at the following concentrations: iron, 5·0 p.p.m.; zinc, manganese and copper, 2·0 p.p.m.; and molybdenum, 1·0 p.p.m.

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The strain of *S. griseus* used was supplied through the courtesy of Boots Pure Drug Co. and was a selected variant of the original Waksman 4 strain. To avoid carry-over of metals in the spores of the inoculum, felts were grown on 50 ml. of purified medium in 500 ml. Hysil or Pyrex flasks, and after aseptically removing the culture liquor, were shaken for 5 min. with 50 ml. of sterile double-distilled water. This suspension was filtered through sintered glass of porosity 3 before use and 1·0 ml. used to inoculate each flask.

The experiments were carried out with 20 ml. of medium in 100 ml. conical Hysil or Pyrex flasks sterilized at 15 lb./sq.in. for 15 min. All cultures were incubated at 28° for 8 days.

The assay of antibiotic activity was carried out by the cylinder-cup method using *Bacillus subtilis* N.C.T.C. 7241 as test organism. All figures given are the average of eight ring assays of three replicate cultures. Growth was estimated as total dry-weight mycelium per flask. All figures are the average of three replicate cultures.

RESULTS

The effect of zinc on growth and streptomycin production

Treatment of the culture medium with dithizone removes copper as well as zinc. Copper was therefore added as $CuSO_4.5H_2O$ to the purified medium to give a concentration of 2 p.p.m. of copper, and graded amounts of zinc as $ZnSO_4.7H_2O$ added to a series of replicates.

Fig. 1 shows that zinc is indispensable for the growth of *S. griseus*. With no added zinc the purified culture solution supported little growth. When zinc

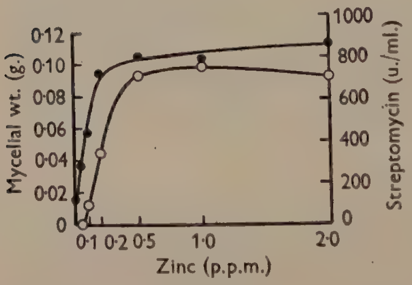

Fig. 1

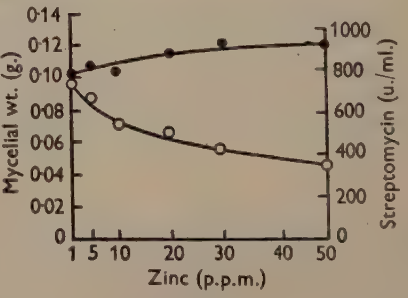

Fig. 2

Fig. 1. The effect of zinc deficiency on growth (●——●) and streptomycin production (○——○).

Fig. 2. The effect of high concentrations of zinc on growth (●——●) and streptomycin production (○——○).

was partially deficient (0·1–0·2 p.p.m.) the mycelial felt was thin, had little aerial mycelium and no spores. Lysis occurred in submerged areas of the felt. Maximum development of aerial mycelium and of spores was not obtained with less than 1·0 p.p.m. of zinc; larger amounts of zinc merely accelerated the rate of growth to a slight extent.

The optimum concentration of zinc for antibiotic production was 1·0 p.p.m. At concentrations above 1·0 p.p.m., zinc depressed antibiotic production and at 50 p.p.m. the titre was only half that at the optimum level. The results shown in Figs. 1 and 2 agree with those of Woodruff (1947) and Spilsbury (1948), who reported that concentrations of zinc above 10 p.p.m. depressed antibiotic production. Our results, however, indicate that the optimum concentration is relatively low and that quantities in excess of 1·0 p.p.m. are unfavourable for antibiotic production.

The effect of iron on growth and streptomycin production

The culture medium was purified with oxine and 2·0 p.p.m. of zinc, manganese and copper and 1·0 p.p.m. of molybdenum added to restore the concentrations of these metals. Iron, as $FeSO_4.7H_2O$, was added in graded amounts to a series of replicates.

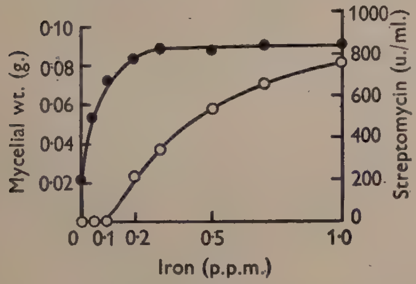

Fig. 3

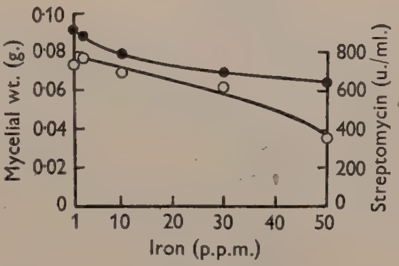

Fig. 4

Fig. 3. The effect of iron deficiency on growth (●——●) and streptomycin production (○——○).

Fig. 4. The effect of high concentrations of iron on growth (●——●) and streptomycin production (○——○).

Fig. 3 shows that iron is essential for the growth of *S. griseus*. Iron deficiency, unlike zinc deficiency, does not result in abnormal growth. Sporulation occurred even with the lowest concentration of iron and increased up to 0·5 p.p.m. The effect of higher concentrations of iron is shown in Fig. 4. Beyond 1·0 p.p.m. growth decreased steadily and the spores became pink, giving a pronounced tint to the whole felt which deepened to a rose pink at 50 p.p.m.

At low concentrations the effect of iron on antibiotic production did not run parallel with the effect on growth (Fig. 3). While with 0·1 p.p.m. of iron growth was *c.* 80 % of maximum, antibiotic production had not reached a measurable value. At concentrations between 0·2 and 1·0 p.p.m., iron showed a specific effect on antibiotic production. Over this range, growth was almost unaltered, whereas antibiotic production rose from a very low level to almost the maximum value. Whereas 0·3 p.p.m. was sufficient for optimum growth, optimum antibiotic production required between 1·0 and 2·0 p.p.m. of iron. With higher concentrations of iron, growth and antibiotic production were both appreciably reduced. With 50 p.p.m. of iron, growth was *c.* 75 % of the maximum and streptomycin production only 45 % of the maximum (Fig. 4).

The effect of copper on growth and streptomycin production

Copper was found to be a difficult element to remove from the culture solution and to guard against as a contaminant. The medium was extracted with dithizone, and sufficient $ZnSO_4.7H_2O$ added to restore the concentration of 2·0 p.p.m. of zinc. To decrease the carry-over of copper in the spores of the inoculum the latter was passaged on a purified medium and inoculation carried out by means of an all-glass spray. Using this method of inoculation 0·1 ml. spore suspension per flask was sufficient, as compared with 1·0 ml. used when inoculated by a pipette.

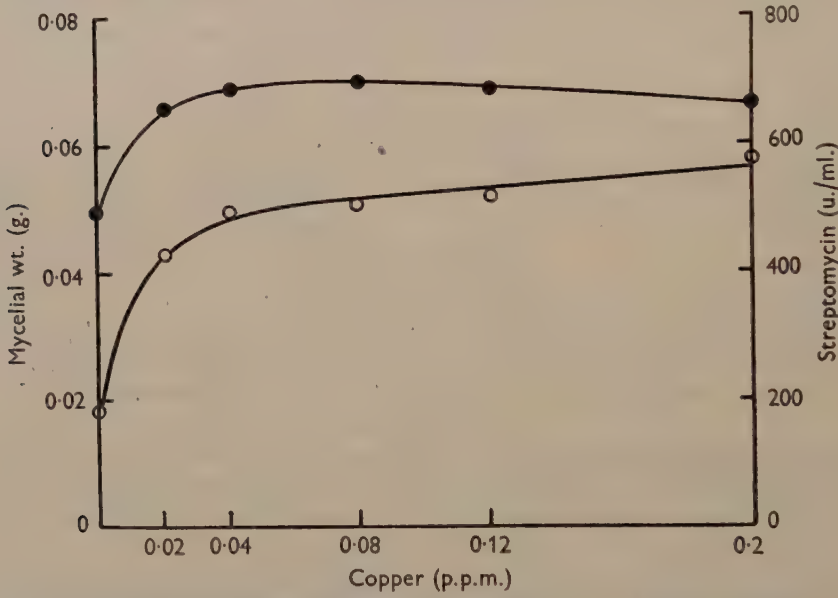

Fig. 5. The effect of copper deficiency on growth (●——●) and streptomycin production (○——○).

The effect of copper deficiency on growth and antibiotic production is shown in Fig. 5. Although the deficiency in growth produced in these experiments from a lack of copper was not so marked as with the lack of zinc or iron, it is clear from Fig. 5 that the metal is nevertheless essential. Cultures without added copper were only 72% of the weight of normal cultures. If a completely copper-free culture could be realized it is almost certain that no growth would occur. Copper deficiencies were indicated also by the colour of the spores, which changed from the normal dirty white through grey to dark brown-grey as the copper deficiency became more acute.

Without added copper the titre was less than 30 % of the maximum value (Fig. 5). The minimum amount of copper for optimum antibiotic production was approximately the same as for growth, i.e. 0·05 p.p.m., although titres did tend to increase slightly with increasing amounts of copper up to as much as 1·0 p.p.m. Above 1·0 p.p.m., concentrations of copper up to 50 p.p.m. showed little effect on either growth or antibiotic production. Compared with the results of Spilsbury (1948), our results indicate that the copper requirements of *S. griseus* are relatively small and that no marked inhibitory effects occur at concentrations of even 50 p.p.m. Providing 0·05 p.p.m. of copper was present this metal appeared to have little effect on *S. griseus*.

The effect of manganese on growth and streptomycin production

When the medium was purified by means of oxine and the trace elements other than manganese returned, the mycelial growth produced after spray inoculation showed neither deficiency of growth nor of antibiotic production in

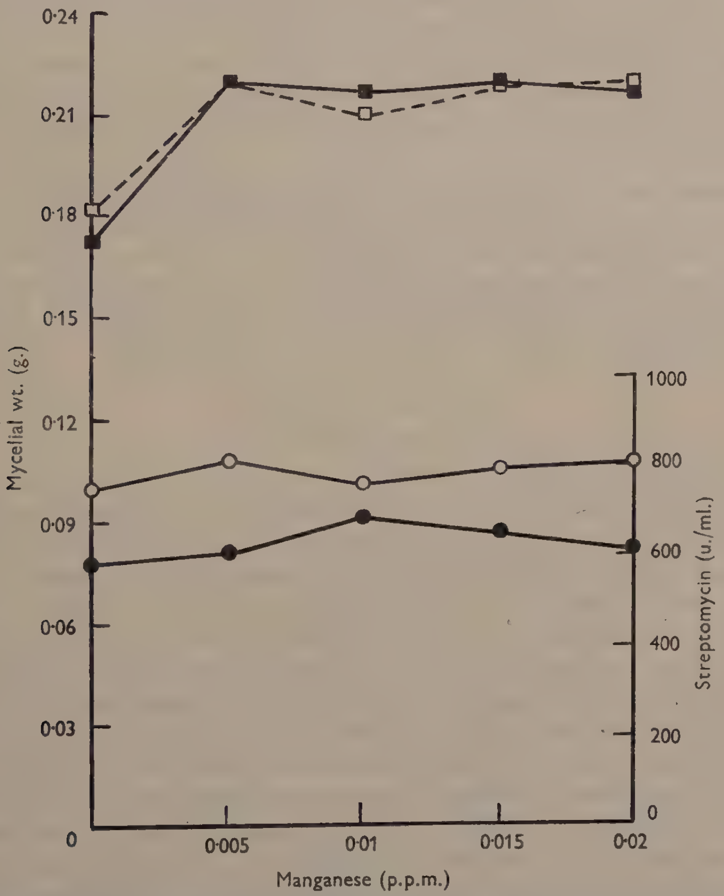

Fig. 6. The effect of manganese on the growth of *S. griseus* (●——●) and on streptomycin production (○——○) and on the growth of *A. niger* in the same medium (■——■) and with sterilized *S. griseus* inoculum present in the medium (□----□).

any of the experiments. The spore suspensions used were prepared from felts which had been grown through three passages on such a purified medium. The purified medium was also tested by inoculation with *Aspergillus niger* which had been grown on a purified medium containing only 0·01 p.p.m. manganese. Marked manganese deficiencies were at once obtained with *A. niger*. With no added manganese the cultures were completely devoid of spores and consequently white, in contrast with the normal jet-black appearance, and the mycelial weights of these deficient cultures were decreased to *c.* 80 % of normal. It was quite evident that the purified medium did not contain sufficient manganese for the normal growth of *A. niger* yet when inoculated with *S. griseus* neither growth nor streptomycin production were diminished. The growth of *A. niger* showed the same degree of deficiency when the *S. griseus* inoculum was added to the purified medium before sterilization. This experiment, together with the fact that the inoculum was passaged three times on the purified medium, suggested that the manganese content of the inoculum was not appreciable. Fig. 6 shows these effects of manganese on the growth of *A. niger* and *S. griseus* and also on streptomycin production. The absence of any decrease in streptomycin production when no manganese was added was more remarkable than the absence of growth deficiency, since with the other trace elements studied streptomycin production suffered more than growth as the metal became deficient.

Manganese has been shown to be required by a wide range of living organisms and it seems not unlikely that it is essential for life in general. If manganese is in fact essential for the growth of *S. griseus* the minimum amount required would appear to be extremely small and certainly less than 0·005 p.p.m. Concentrations of manganese up to 50 p.p.m. had little effect on growth or antibiotic production other than to depress the latter slightly.

DISCUSSION

With the exception of iron, none of the trace elements studied here showed any marked effect on antibiotic production other than by way of altered growth, and providing a sufficient concentration was present for growth, antibiotic production was also optimum. With regard to iron, however, normal growth was not itself sufficient to achieve optimum antibiotic production. Some mechanism would appear to exist in streptomycin formation which requires approximately five times the quantity of iron sufficient for growth alone.

The apparently conflicting reports which have been published on the effects of trace elements on streptomycin production may be largely due to the use of complex media which may contain considerable quantities of trace elements. Such media may well furnish sufficient trace elements for optimum growth without any intentional addition. Complex media containing natural products such as meat extract are more likely to contain toxic quantities of trace elements than to be deficient. Fig. 2 shows that this might very well occur in the case of zinc where quantities greater than 1·0 p.p.m. inhibited streptomycin production in the medium used.

The fact that zinc, iron and copper are essential for the growth of *S. griseus* agrees with the findings for a wide range of green plants, fungi and bacteria, and lends support to the probability that these elements are indispensable to life in general. The apparently minute manganese requirement of *S. griseus* is remarkable, and it would be of interest to know whether this is characteristic of other actinomycetes.

The authors wish to express their gratitude to Boots Pure Drug Co. Ltd. for the award of a Fellowship to one of us (G.N.R.) to enable this work to be carried out.

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The Isolation of Organisms of the Pleuropneumonia Group from Dogs

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SUMMARY: The finding of a pleuropneumonia-like organism associated with infertility and epididymitis in a dog led to the examination of throat and vaginal swabs from all the animals in a breeding kennel. Pleuropneumonia-like organisms were isolated from the majority of samples. Strains differed in colonial appearances, and a classification into types based on these differences was confirmed by the serological demonstration that each type was antigenically distinct. Nearly all the strains belong to one of three types, provisionally called α, β and γ. Each type was regarded as a distinct species of the pleuropneumonia group, and its biological properties were studied.

The semen of a dog admitted for investigation of sterility and epididymitis was used for the artificial insemination of two bitches in the breeding kennel. Two days later a stud dog was mated with one of the bitches. Soon afterwards the semen of this dog showed evidence of impaired fertility and inflammation of the epididymis was suspected. A bacteriological examination of the semen yielded positive cultures of pleuropneumonia-like organisms. Similar organisms were cultivated from the semen of the dog originally brought for investigation and from a vaginal swab from the bitch which had been inseminated. These findings suggested that an organism of the pleuropneumonia group had been found which was pathogenic for the canine genital tract. With the idea of investigating the distribution of the organism, cultures were made from vaginal swabs or semen, and from throat swabs obtained from all the animals in the kennels. Pleuropneumonia-like organisms were isolated from the majority of specimens, but the cultural appearance of the strains varied so much as to suggest that several different members of the pleuropneumonia group inhabited the genital and respiratory tracts of these dogs. In only a few of the cultures did the colonies resemble those of the strains isolated originally from the two dogs and one bitch. The investigation described in this paper, which confirmed that the strains belonged to several different species of the pleuropneumonia group, enabled each strain to be identified. Unfortunately, however, owing to the time taken to accomplish this and to the difficulty in maintaining the many strains meanwhile, it was not possible to investigate pathogenicity.

METHODS

Vaginal swabs were taken through a glass speculum, after thorough cleansing of the external genitals with spirit. Cultures were made on a special selective medium (Edward, 1947). Two agar plates and one bottle of semi-solid medium were inoculated with each sample; one plate was incubated aerobically and the other anaerobically. The plates were examined after 3 and 4 days' incubation, using a binocular dissecting microscope (× 10) and slightly oblique transmitted light. When colonies of pleuropneumonia-like organisms were seen, subcultures were made from single colonies; on some plates there appeared to be more than one type of colony, and subcultures were made from each type.

For serological investigation suspensions of organisms were prepared from cultures in broth enriched with horse serum or human ascitic fluid. Antisera were made in rabbits by repeated inoculation with the deposits of cultures grown in rabbit serum broth. Certain strains did not grow in rabbit serum broth, but good growth was obtained when cholesterol (0·1 mg./ml.) was added to the medium as a finely dispersed suspension (Edward & Fitzgerald, 1951). The techniques for the agglutination and complement-fixation tests were the same, except for a few modifications as those used previously in the study of bovine genital strains (Edward, 1950 *a*). The agglutination tests were incubated at 52° for 6 hr. and then at 37° overnight, and readings were made at 4, 6 and 20 hr. In the complement-fixation tests two full haemolytic units of complement were used.

RESULTS

Isolation of strains

Swabs from vagina and throat of thirty-nine bitches were examined; from only four animals were the cultures from both swabs negative for pleuropneumonia-like organisms. From fifteen bitches, pleuropneumonia-like organisms were grown from both vagina and throat; from six, vaginal swabs were positive and throat negative, and from fourteen, throat was positive and vagina negative. The colonial appearances of the various strains differed markedly. These differences, first apparent in the primary culture, were maintained in the early subcultures. Some swabs yielded at least two types of colony, which bred true on subculture. These observations suggested that several different members of the pleuropneumonia group inhabited the throat and genital tracts of these dogs. In a preliminary classification of the strains based on their colonial appearances three types were recognized; for convenience they were designated by the first three letters of the Greek alphabet.

α-Strains. These strains, although not the most numerous, were of special interest. Of this type were the three strains which had been isolated from the infertile dog, from the bitch mated with it and from the stud dog apparently infected by subsequent mating with the infected bitch. The α-strains were distinguished by having a coarsely reticulated colony. When viewed by transmitted light the thick dark markings suggested the presence of large globules inside the colony (Pl. 1, fig. 1). In young colonies often only the centre was heavily marked, the periphery being conspicuously translucent with a smooth surface resembling a drop of water. The colonies were otherwise typical of the pleuropneumonia group, except that the central spot tended to be hidden by the coarse markings. Most of the α-strains were isolated from semen or vaginal material. The colonies of these genital strains were small and about the same size as those of human and bovine genital strains. There were also a few strains, isolated from throats, whose colonies had similar markings, but were larger.

β-Strains. These strains were the most common and were isolated from both throat and genital tract. The colonies were large and the central spot, which is so characteristic a feature of colonies of the pleuropneumonia group, was absent or poorly developed. These characters were most noticeable on primary isolation. On the original plates the colonies were so large, that, having no obvious central spot, it was difficult to recognize them as belonging to the pleuropneumonia group. However, they had the typical translucency, fine surface markings, and other characters of pleuropneumonia-like organisms (Pl. 1, fig. 2). Some strains, after 3 days' incubation, formed flattened colonies with irregular margins. With repeated subculture the special features characterizing the β-strains tended to disappear, more quickly with some strains than with others, so that they became indistinguishable from other members of the pleuropneumonia group.

γ-Strains. A number of strains, isolated from vagina and throat, when grown on media enriched with horse serum yielded surface growths which became covered by a peculiar 'pearly' film; these were termed 'γ'-strains. Under the dissecting microscope the film was crinkled, covering the colonies and spreading between them. There were also numerous small black dots in the substance of the medium just beneath and around the colonies. These appearances were identical with those described in cultures of two other members of the pleuropneumonia group, the P organism from the bovine genital tract and the L3 organism from mice (Edward, 1950*a*, *b*). With the γ-strains, however, the film appeared earlier, often by the third day of incubation, and was better developed than with the other organisms. Colonies of the γ-strains otherwise had no distinctive characters (Pl. 1, fig. 3).

From one vaginal swab a pleuropneumonia-like organism was obtained on a plate incubated anaerobically, the corresponding aerobically incubated plate remaining sterile. Attempts at subculture aerobically and anaerobically failed. It was thus impossible to establish whether this strain was an obligatory anaerobe and different from the others.

Dienes (1947*a*, *b*) called attention to a possible danger in using penicillin to provide a selective medium for the isolation of pleuropneumonia-like organisms. Pleuropneumonia-like forms can be developed as variants from various bacteria on media containing penicillin. In view of the large number of strains isolated here from dogs by the use of media containing penicillin, it was necessary to exclude the possibility that the organisms had been derived from bacteria in the specimens taken. In making the cultures penicillin was always applied only to half of the agar plate. Both halves were inoculated, and it was noted that colonies of the pleuropneumonia-like organisms appeared on the penicillin-free part of the plate, except when they were overgrown or inhibited by bacteria. A number of strains which were subcultured directly on to media free from penicillin retained their characters and did not become bacillary in form even though subculture was repeated on penicillin-free media.

The preliminary three classes of strains, each possibly a different species of the pleuropneumonia group, were further investigated by cultural and serological methods. In order to decrease the number of strains to be examined, strains with colonies exactly similar to those of another strain were discarded. There remained thirty-four strains from which suspensions were prepared for serological study. Typical α-, β- and γ-strains were included, together with strains whose identification on colonial appearance was doubtful. The serological investigation, which provided most information and confirmed the original classification, is reported first.

Serological investigation

Antisera were prepared in rabbits against two representative α-strains, strain no. 48 isolated from the vagina, and strain no. 21 which had come from the throat and gave larger colonies. Strains nos. 55 and 51 were chosen as typical β-strains for the production of antisera. The former had been isolated from the throat and gave smooth growth in fluid and semi-solid media; the latter, isolated from the vagina, gave a more granular growth, although good suspensions were obtained for agglutination. Antisera were also made against the γ-strains, nos. 27 and 35.

In cross-agglutination tests the antisera to the β-strains, nos. 51 and 55, agglutinated both homologous and heterologous organisms to a dilution of at least 1/2000. One or other, or both, these antisera agglutinated to a significant titre twenty-one other strains, most of which had previously been identified as β-strains, the identity of the rest being uncertain. Although with two strains, nos. 33 and 52, the titres were low, the others were agglutinated to at least one-quarter of the full titre.

The antiserum to strain no. 48 agglutinated the homologous strain, and strain no. 28 to a dilution of 1/1024 and strains nos. 34 and 46 to 1/256. These four strains with α-type colonies had been isolated from the genital tract. They were not agglutinated by the antiserum prepared against strain no. 21 which had been isolated from the throat and had given larger colonies of the α-type. This antiserum did not agglutinate any of the other strains. Antisera to the γ-strains, nos. 27 and 35, agglutinated each other and also two other strains, provisionally identified as γ, to at least one-quarter of the full titre. In these tests agglutination by at least one antiserum was obtained with all except four strains, suspensions of three of them being unsatisfactory because of granularity. Little cross-agglutination occurred between the α-, β- and γ-strains; it did not exceed a dilution of 1/64 and was most often noted with the two γ-antisera. Sometimes in low dilutions of serum there was a coarse flocculation; this was disregarded as being non-specific. Thus in some tests readings were not obtained in the low dilutions; dilutions of serum less than 1/16 were not tested.

The antisera were tested for their ability to fix complement in the presence of the homologous organism. Strain no. 27 antiserum fixed complement to the same titre as the agglutinating titre; the titres for the other antisera were 1/4 or 1/8 of the agglutinating titres. Complement-fixation tests carried out with a number of the strains confirmed the findings with the agglutination reaction. The genital α-strains, the β- and γ-strains and strain no. 21 appeared to be distinct antigenically. Strain no. 27 antiserum fixed complement to

Table 1. *Results of agglutination and complement-fixation* (C.F.T.) *tests*

Strain no.	Provisional identification*	Antiserum to strain 51		Antiserum to strain 55		Antiserum to strain 48		Antiserum to strain 27		Antiserum to strain 35, agglutination titre	Antiserum to strain 21	
		Agglutination titre	C.F.T. titre	Agglutination titre	C.F.T. titre	Agglutination titre	C.F.T. titre	Agglutination titre	C.F.T. titre		Agglutination titre	C.F.T. titre
51	β	2048	256	2048	32	64	<2	32	8	32	32	<2
55	β	2048	16	4096	512	<16	<2	64	32	<32	<16	<2
58	β	2048	256	512	64	<32	—	<32	—	<32	32	—
57	β	1024	128	1024	128	<64	—	64	—	<32	32	—
42	β	2048	128	256	4	64	<2	64	8	<32	<64	2
11	?β	2048	16	256	256	<32	—	<32	—	<32	<16	—
15	?β	1024	8	512	256	<32	—	<32	—	<64	<32	—
18	β	1024	32	2048	256	<32	—	<32	—	<32	<32	—
30	?	1024	32	2048	512	<32	—	<32	—	<32	32	—
40	β	512	32	1024	128	<64	—	<32	—	<32	<32	—
47	β	2048	8	4096	128	<32	—	32	—	<16	<16	—
49	?β	2048	32	4096	128	<32	—	64	—	<32	<32	—
50	β	2048	64	512	128	<16	—	<16	—	<32	<16	—
4	?	128	—	1024	128	<32	—	16	—	32	<32	—
9	β	2048	—	1024	—	<16	—	<32	—	<32	<16	—
12	?β	1024	—	512	—	<32	—	64	—	<32	32	—
17	?β	2048	—	1024	—	<64	—	<64	—	<64	<64	—
22	β	2048	—	1024	—	<32	—	64	—	<32	<16	—
53	β	512	—	1024	—	<32	—	32	—	<32	<32	—
52	β	32	32	256	512	<64	<2	<32	8	<64	<32	<2
33	?β	256	64	64	64	16	<2	16	8	<16	<16	<2
39	?	<32	16	<32	>64	<32	<2	<16	16	<16	<32	<2
48	α	<16	2	<16	4	1024	256	<16	32	<32	<16	4
34	α	<16	<2	<16	<2	256	128	<32	8	<16	<32	<2
28	α	<32	—	<32	—	1024	—	<64	—	<64	<32	—
46	α	<32	—	<32	—	256	—	32	—	<16	<16	—
27	γ	<32	<2	<32	4	<32	4	2048	2048	2048	<16	<2
35	γ	<16	—	<16	—	<16	—	1024	—	2048	<16	—
20	γ	<32	—	<16	—	<16	—	512	—	1024	<64	—
44	γ	<32	—	<32	—	<128	—	512	—	128	<64	—
21	α	<32	<2	<32	8	<32	<2	<32	32	<32	2048	256
59	?	G	<2	G	<2	G	<2	G	8	G	G	<2
60	?α	G	<2	G	<2	G	<2	G	8	G	G	<2
45	α	G	—	G	—	G	—	G	—	G	G	—

* Provisional identification based on colonial appearances.
G signifies that the suspension was unsuitable for the agglutination test because of granularity.

a dilution of 1/32 with strains nos. 55 and 48, but this was only $\frac{1}{64}$th of the full titre; otherwise cross-reactions were insignificant. The inagglutinable strain no. 39 was identified as a β-strain, as it fixed complement to a dilution exceeding 1/64 with strain no. 55 antiserum. Two of the three strains which had been too granular to provide agglutinable suspensions were tested and did not fix complement with any of the antisera.

It has already been noted that not every strain was agglutinated to a full titre by the corresponding antisera. This might be partly explained by poor agglutinability of certain suspensions. However, some β-strains were agglutinated to full titre by one β-antiserum and to a low titre only by the other β-antiserum. As this suggested that antigen differences might occur among the β-strains, agglutinin-adsorption tests were carried out.

Table 2. *Results of agglutinin-adsorption experiments*

	51 antiserum			55 antiserum		
Antigen	Unadsorbed	Adsorbed with 55	Adsorbed with 12	Unadsorbed	Adsorbed with 51	Adsorbed with 12
51	2048	2048	2048	2048	< 64	256
55	2048	< 32	2048	4096	4096	4096
12	1024	256	< 64	512	256	< 64
4	128	32	128	512	512	256
42	2048	2048	512	256	< 128	128
17	2048	1024	—	1024	128	128
15	1024	< 32	—	512	256	512

Strain no. 51 antiserum after adsorption with strain no. 55, agglutinated the homologous organism to full titre, although no longer capable of agglutinating strain no. 55. Similarly, agglutinins for the homologous organism were not affected by adsorbing strain no. 55 antiserum with strain no. 51. The adsorbed sera were shown to agglutinate a number of other β-strains, usually to the original titre, but sometimes to a lower titre. Agglutinins for strain no. 15 were removed completely by adsorbing strain 51 antiserum with strain no. 55. Similar results were obtained by adsorbing the antisera with strain no 12; the adsorbed sera agglutinated the suspensions tested to the original or slightly lower titres. The β-strains thus appear to have a complex antigenic structure which could not be analysed further.

Although cross-agglutination to approximately full titre occurred between strains nos. 51 and 55, the complement-fixing titre for the heterologous strain was only $\frac{1}{16}$th that for the homologous. Several other β-strains in complement-fixation tests gave a high titre with one antiserum and a low titre with the other. The complement-fixation test thus appeared to be more specific than the agglutination reaction.

The serological results closely confirmed the original classification of strains based on colonial appearances. All those strains with relatively small colonies, classified as α and isolated from the genital tract, resembled each other antigenically and differed from other strains. Strains with a β-type of colony, together with a number of strains which had not been definitely identified, formed a second antigenic group. Similarly strains classified as γ because they produced a characteristic film and precipitate in horse serum media were also alike serologically. Only four strains, isolated from the throat, appeared not to belong to any of these three groups. Strain no. 21 was shown to be antigenically different from the α-, β- or γ-strains; the other three strains resembled each other in yielding granular growths in broth cultures and appeared to be unrelated serologically to the other strains. Colonies of strain no. 21 and of one, at least, of the granular strains were of the α-type, although larger than those of the genital strains. Thus in future the term 'α' will be restricted to the group of genital strains.

Cultural characters

It appeared from previous observations that differences in biological properties could be used for identifying members of the pleuropneumonia group (Edward, 1950*b*). These observations were made on laboratory strains which had been maintained by subculture for years. It has now been possible to determine whether an examination of the same properties might assist in the identification of freshly isolated canine strains.

None of the canine strains produced visible growth at room temperature or on media devoid of serum. Representative α-, β- and γ-strains grew as well anaerobically, as aerobically. In the earlier examination of members of the pleuropneumonia group the appearance of growth in semi-solid and fluid media had proved most helpful in identification. There was a sharp distinction between those strains which grew in semi-solid media as well-circumscribed irregular masses and in broth as a deposit leaving the supernatant clear, and those which gave a uniform smooth growth. The recently isolated canine strains were more difficult to classify as granular or smooth. Only a few β-strains, such as strain no. 55, gave a typically smooth growth. The other strains appeared granular, but it was noted that with repeated subculture the tendency to granularity diminished. Many strains were neither definitely granular nor completely smooth. As already noted, the growth of only three strains was so granular that satisfactory agglutinating suspensions could not be obtained.

Fermentation of glucose was not detected with any strain. Haemolysis was noted around colonies on agar plates containing horse serum and horse erythrocytes. There were wide zones of clearing around colonies of β-strains. Less haemolysis was produced by γ-strains; only very slight clearing was produced by α-strains. The unidentified strains, nos. 21, 45, 59 and 60, were also haemolytic. Reduction of methylene blue was obtained with some, but not all, young cultures of β- and γ-strains. In a few tests reduction by α-strains was not noted. Strain no. 21 and the two β-strains, nos. 51 and 55, grew well on media enriched with rabbit serum instead of horse serum. The α-strain, no. 48, and all four γ-strains tested did not at first grow at all, or growth was very poor, on rabbit serum plates, and a long period of adaptation was necessary before they grew satisfactorily with rabbit serum instead of horse serum.

In these tests no clear-cut differences were obtained between the various strains. Thus tests which had been useful in the identification of other members of the pleuropneumonia group were of no help in differentiating the canine strains. Identification of the latter, except by serological means, depended on the colonial appearances already described. The peculiar film and precipitate formed in media containing horse serum distinguished γ-strains; they were not noted with other strains and were still formed by γ-strains after maintenance in subculture for months. The characteristic colonial appearances of β-strains were quickly lost on subculture; with some strains they were less evident even in the first subculture. It was for this reason that a number of β-strains were not identified with certainty, except by their serological reactions. After subculture the colonies of some β-strains showed a tendency to vacuolation, so that they resembled α-colonies. The α-strains maintained their typical colonial appearances for a few subcultures, but strains maintained in this way for months were no longer characteristic.

Distribution of strains among the bitches

Each animal in the kennels was swabbed once. Pleuropneumonia-like organisms were isolated from approximately half the vaginal swabs and from three-quarters of the throat swabs (Table 3). From three vaginal swabs and from eight throat swabs two different types of organism were isolated; from one vaginal swab three types, α, β and γ, were grown. The number of isolations of each type of organism is shown in Table 3.

Table 3. *Distribution of strains*

Material	No. of samples examined	Samples positive		No. of strains isolated			
		No.	%	α	β	γ	Other strains
Vaginal swabs	44	24	54·5	10	10	6	2
Throat swabs	39	29	74·4	0	23	9	5

DISCUSSION

Sabin (1939) and Sabin & Johnson (1940) showed that several members of the pleuropneumonia group, each with different pathogenic properties, inhabited the upper respiratory tract of mice. Edward (1950*a*) found that strains isolated from the genital tract of cattle belonged to one or other of two species, recognized by their cultural properties. The findings in dogs therefore provided another illustration that more than one member of the pleuropneumonia group may reside in the same situation. The different types of canine strain were distinguished by their colonial appearances and, as the differences were well-marked in the primary cultures, it was possible by inspection to establish whether more than one type of organism was present in a culture. Serological investigation largely confirmed the classification of strains based on colonial appearances, and by demonstrating that each type of strain was distinct antigenically suggested that they were separate members of the pleuropneumonia group.

Pleuropneumonia-like organisms were first isolated from dogs by Shoetensack (1934, 1936*a*, *b*); they were found in secretion from the upper respiratory tract and in the tissues of animals suffering from canine distemper. Shoetensack regarded them as the cause of this disease, and described experiments in which the inoculation of healthy dogs with cultures apparently produced distemper. His strains differed in their properties and appeared to belong to two groups, or species, named by him *Asterococcus canis* I and *A. canis* II. It has not been possible to obtain cultures of Shoetensack's strains for comparison with those isolated by us. It is also impossible to determine from his original descriptions whether any of our strains are identical with either *A. canis* I or II. He described an opaque halo surrounding colonies of *A. canis* II on horse serum agar, and it is possible that this was the same as the 'pearly' film and precipitate associated with γ-strains. Shoetensack, however, stated that an even more marked opacity developed around cultures of *A. bovis*; none of our strains of the latter organism have produced a film or precipitate.

Klieneberger (1938) showed that *A. canis* I and II differed antigenically. She described a dissociation of *A. canis* I into two types of colony, one, the 'coarse', having the characters of the α-type. The α-strains from the genital tract cannot be identified as *A. canis* I on this evidence alone, because certain antigenically different strains from the throat also had α-like colonies. Moreover, after prolonged subculture several other strains showed a tendency to form this coarse type of colony.

The pleuropneumonia group has been classified into a number of species, based on cultural properties, habitat, pathogenicity and antigenic constitution (Sabin, 1941). The differences between the strains isolated by Shoetensack led him to regard them as two different species. Differences between our strains are equally marked and would appear to warrant the assumption that they represent distinct species. Most of these strains belonged to one of three species, provisionally designated by Greek letters to avoid confusion with *A. canis* I and II. An agreed systematic classification and nomenclature for the pleuropneumonia group is very necessary, but should be postponed until more is known about these organisms. We think it is desirable meanwhile to apply provisional labels only to species in order not to prejudice a more systematic nomenclature later.

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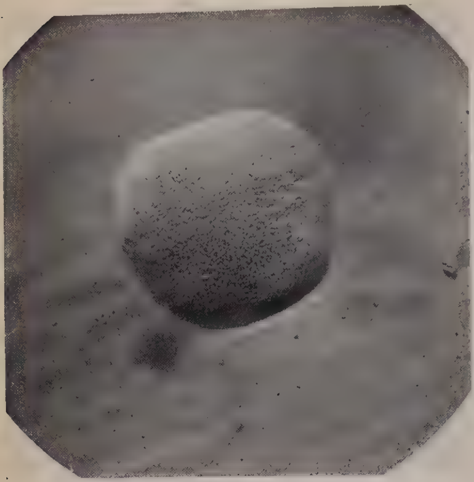

Fig. 1

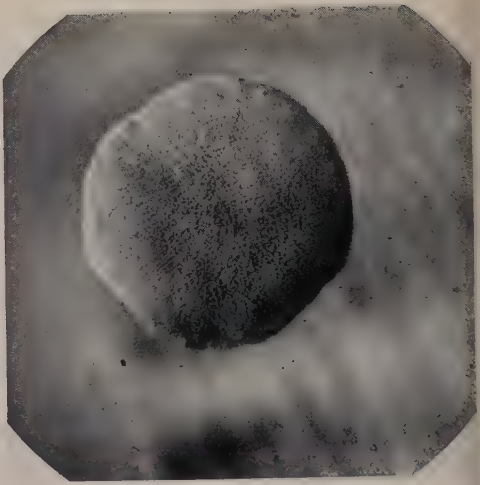

Fig. 2

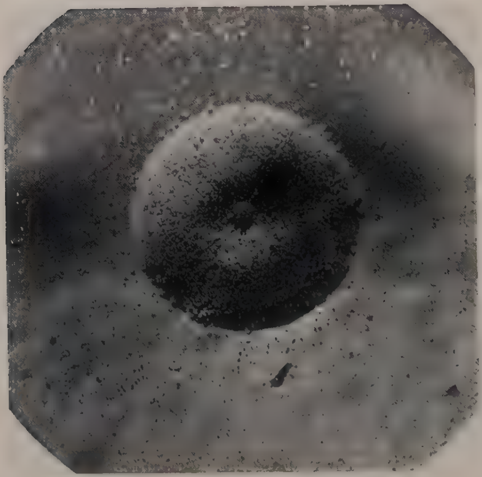

Fig. 3

D. G. ff. EDWARD & W. A. FITZGERALD—PLEUROPNEUMONIA-LIKE ORGANISMS FROM DOGS. PLATE 1

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EXPLANATION OF PLATE

(Colonies viewed by slightly oblique transmitted light. × 100 approx.)

Fig. 1. Strain 45. Typical α-type colony; note coarse marking with vacuolation, the periphery remaining clear.

Fig. 2. Strain 51, a typical β-strain. Note relatively flattened colony, without an obvious central spot.

Fig. 3. Strain 35, a typical γ-strain. Dome-shaped colony with well-marked central spot; note the numerous spots in the medium and the beginning of a crinkled film on the surface of the colony.

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Cholesterol in the Growth of Organisms of the Pleuropneumonia Group

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SUMMARY: Representative strains of the pleuropneumonia group, originally isolated from animals, grew well on a basal medium containing infusion broth, peptone, yeast extract and agar when this was further enriched with horse serum. The serum could be replaced by an ethereal extract of egg-yolk and fractionation of this suggested that cholesterol might be the active substance promoting growth. Growth was obtained when cholesterol (0·01 mg./ml.) was added to the basal medium, together with starch or bovine albumin. Addition of the acetone-insoluble fraction of egg-yolk with cholesterol gave better results, growth of six of the eight strains tested being equal to that on serum agar; the lecithin and kephalin fractions after purification were less effective. No growth was obtained when starch, bovine albumin or the acetone-insoluble lipid was added to the medium without cholesterol. Cholestanol and stigmasterol were as effective as cholesterol in promoting growth. Oleic acid in high concentrations ($>0{\cdot}05$ mg./ml.) inhibited growth. Both lipid and protein fractions of serum appeared to be concerned in its ability to promote growth.

Pleuropneumonia-like organisms, whose smallest viable forms have been shown by ultra-filtration studies to measure *c*. 150–200 mμ., are the smallest micro-organisms which can be cultivated on cell-free media. Their nutritional requirements are therefore of particular interest. Moreover, a study of their metabolism may assist in establishing the relationship of this group of organisms to the ordinary bacteria. Although certain saprophytic members of the group can be cultivated easily on relatively simple media (Laidlaw & Elford, 1936), conditions for the growth of strains isolated from animals, including all the pathogens, are more exacting. In particular, the latter strains differ from the saprophytic ones in needing media further enriched by materials to be found in serum and ascitic fluid. The necessity to add a complex material such as serum complicates the investigation of essential growth requirements and the development of a defined medium.

Not all animal sera are equally effective for growing every strain (Sabin, 1941; Edward, 1950). Of those tested horse serum and human serum (or ascitic fluid) have proved most satisfactory and are commonly used. With rabbit serum some strains grow well; others grow poorly or not at all. It is perhaps possible that organisms of this group exist which cannot be isolated on media at present in use because neither horse nor human serum provides adequate enrichment.

In undertaking a study of growth requirements it was therefore considered desirable to investigate first the material in serum which was essential for growth. The earliest experiments were based on the belief that it was serum protein which was needed (Sabin, 1941; Edward, 1950). No progress was made, however, in attempts to isolate a growth factor by fractionation of the serum protein. The finding that a precipitate, which developed as a result of the growth of certain strains in media enriched with horse serum, consisted of calcium soaps, drew attention to the importance of lipids and thereby suggested a new approach. It was found that pleuropneumonia-like organisms would multiply on a medium in which serum was replaced by an ethereal extract of egg-yolk. This observation led to the work described here.

METHODS

Details are given in Table 1 of the eight strains, each believed to belong to a different species of the pleuropneumonia group, which were used in the investigation. They were maintained as surface cultures by twice weekly subculture on horse serum agar. The medium consisted of 70% (v/v) agar base prepared from ox heart infusion broth with the addition of 1% (w/v) peptone, 10 % (v/v) yeast extract (50 mg. Oxoid/ml.) and 20 % (v/v) horse serum; the reaction was adjusted finally to pH 8·0. Thallium acetate and penicillin were incorporated in the medium to prevent the growth of contaminating bacteria (Edward, 1947). In the experimental media the serum was replaced by saline suspensions of the materials to be tested. The volumes of the suspensions were so adjusted, that, whether one or more was added, the total volume of the additions was 20 % of the final volume of the complete medium.

Plates of experimental media were inoculated by cutting out a small piece of agar bearing growth from a 3-day culture on horse serum medium. This piece was inverted over the plate to be inoculated, smeared across it and afterwards discarded. It was usual to inoculate four strains on the same plate by dividing the plate into quarters. Cultures were examined after 3 and 6 days' incubation at 37° in a moist atmosphere. The degree of growth was assessed by examination with a dissecting microscope (magnification × 10), using slightly oblique transmitted light. When the medium was too opaque for the growth to be seen in this way, a hand-lens was used.

To prepare an ethereal extract of egg-yolk, the yolks of three eggs, which had been separated from the egg-white and washed with saline, were emulsified in 50 ml. of saline. An equal volume of ether was added, the mixture well shaken, centrifuged and the ethereal layer removed. This process was carried out from three to five times and the pooled ethereal extracts, after filtration through paper, were distilled at reduced pressure to remove ether and traces of water. The final product was emulsified in saline. Fractionation of the ethereal extract was carried out by distilling off ether until the total volume was *c.* 100 ml. This solution was then pipetted slowly into twice its volume of cold acetone. After standing at 4° for 60 min. the precipitate of acetone-insoluble material was collected by centrifugation. After washing twice with acetone the precipitate was dried in a vacuum desiccator over sulphuric acid. The supernatant fluid containing the acetone-soluble fraction, after filtration through paper, was evaporated to an oily paste by distillation. Emulsions in saline, suitable for addition to the medium, were made by using a Ten Broeck grinder. Other fatty substances examined during the investigation were emulsified in a similar manner; occasionally gum acacia was used to increase the stability of the emulsion.

To prepare finely dispersed aqueous suspensions of cholesterol, 100 mg. were dissolved in 2 ml. of warm ethanol. This solution was ejected into 100 ml. of distilled water at *c.* 80°, using a Pasteur pipette drawn out to a fine capillary. It was essential to introduce all the solution with the point of the pipette below the surface of the water. Crystallization from the ethanolic solution was prevented by keeping both it and the pipette warm. Many excellent suspensions with practically all the cholesterol in a finely dispersed state were prepared in this manner, but the method was not always successful, and several attempts were sometimes necessary before a satisfactory suspension was obtained. It was important to use freshly distilled water of neutral reaction; suspensions rapidly flocculated with a slight change in reaction to the acid side. Flocculation did not occur when the suspension was added to the basal medium. Suspensions of cholesteryl esters and of other sterols were prepared in the same way.

Table 1. *Details of strains used for the investigation*

Strain	Details
PP3278	A strain of *Asterococcus bovis**
L4	Isolated from rats; produced arthritis and abscesses*
M46	Isolated from mice suffering from infectious catarrh*
B2	Isolated from the bovine genital tract; a P strain*
H17	Isolated from the human genital tract*
C27	Isolated from a dog; a γ-strain†
C48	Isolated from a dog; an α-strain†
C55	Isolated from a dog; a β-strain†

* Strains investigated and described by Edward (1950).

† For details of the isolation and behaviour of these strains, see Edward & Fitzgerald (1951).

RESULTS

Growth with lipid fractions of egg-yolk

Each of the eight representative strains was subcultured on the basal medium to which had been added varying amounts of a saline suspension of egg-yolk. All except one strain grew well with each of the concentrations of yolk tested (5–0·25 %); 0·25 % (v/v) appeared to be about the lowest effective concentration. The strain of *Asterococcus bovis* grew poorly over the whole range of concentrations. When an ethereal extract of yolk, resuspended in saline to the original volume of the yolk, was added instead of whole yolk, six of seven strains tested grew well with concentrations between 5 and 1 % (v/v), with 0·5 % (v/v) growth was poor. There was only a trace of growth of *A. bovis.*

An ethereal extract of yolk was separated into two fractions by precipitation with acetone. The acetone-insoluble fraction (subsequently referred to as acetone-insoluble lipid AIL) was tested without further purification for its ability to promote growth. In a number of experiments no growth of any strain was obtained by adding amounts between 4 and 0·4 mg./ml. to the basal medium. All the strains, except *A. bovis*, grew when the acetone-soluble fraction, resuspended in saline to the original volume of the yolk, was added at concentrations between 5 and 1% (v/v). The growth, however, of some strains was poor and appeared to be improved by adding AIL (1 mg./ml.) in addition to the acetone-soluble fraction.

Some of the acetone-soluble fraction was hydrolysed with ethanolic KOH and the unsaponifiable material extracted with ether. Growth of strains C27 and L4 was obtained when the unsaponifiable material was incorporated in the medium together with 1 % soluble starch. On media containing the unsaponifiable material and no starch, strain L4 grew, but strain C27 did not. Thus the factor promoting growth appeared to be contained in the unsaponifiable part of the acetone-soluble fraction of egg-yolk. Since this largely consisted of sterols, especially cholesterol, experiments were continued to discover whether a purified preparation of cholesterol would promote growth.

Promotion of growth by cholesterol

Numerous attempts were made to grow the strains in the basal medium (without serum) by adding cholesterol (0·1 mg./ml.) as a finely dispersed aqueous suspension, prepared as described above. In a few experiments growth of certain strains was obtained. Strain C27 appeared to be the easiest to grow, but in only four out of twenty-two experiments was growth good or moderate. More often growth was only noted on that part of the plate which had been inoculated most heavily; it had probably been assisted by substances carried over in the inoculum. Even when good growth was obtained the colonial appearances were atypical; the outlines of the colonies were irregular and central spots, so typical a feature of pleuropneumonia-like organisms, were absent. Improved results were obtained by incorporating 1 % soluble starch with the cholesterol in the medium. In five out of seven experiments strain C27 grew satisfactorily. Growth of certain other strains was obtained in some, but not in all experiments. Growth tended to be slow in appearing and the colonies were atypical. *A. bovis* failed to grow with cholesterol alone or with cholesterol and starch.

The beneficial effect of starch in some bacteriological media is now believed to be partly due to its ability to bind fatty acids. The effect of adding bovine albumin, fraction V (Amour Laboratories), also used in media because of its ability to bind fatty acids (Davis & Dubos, 1947), was tried. On a medium containing cholesterol and 5 mg. bovine albumin/ml., strain C27 grew at least moderately well in fourteen out of fifteen tests. Strain L4 grew equally as well, and nearly as good results were obtained with strains B2, H17 and M46. The colonial appearances were typical, but it was noted that growth was often slower in appearing than on horse serum agar. *A. bovis* grew satisfactorily on this medium in only one of nineteen tests; growth of strains C48 and C55 also was poor and occurred in only a minority of tests.

In the experiments with fractions obtained from egg-yolk by acetone precipitation it had been noted that, although growth was obtained with the acetone-soluble fraction, it was improved when the acetone-insoluble fraction (AIL) was present in the medium in addition. When AIL was incorporated in the basal medium with cholesterol in concentrations from 4 to 0·4 mg./ml., excellent growth of strain C27 was obtained in every one of fifteen tests. Strains L4, B2, H17, M46 and C55 also grew as well as they did on horse serum agar; there was no delay in the appearance of growth and the colonies were typical. This medium, however, was not satisfactory for the growth of *A. bovis* and strain C48. In three tests, made with the same batch of AIL, *A. bovis* grew moderately well, but eleven other experiments were negative.

When either starch, bovine albumin, AIL or a mixture of bovine albumin and AIL was added to the basal medium without cholesterol, no growth of any strain was obtained. It would therefore appear that cholesterol (or some derivative of it; see below, p. 582) was essential for growth.

Certain strains, such as strain C27, will not grow satisfactorily on media enriched with rabbit serum instead of horse serum unless they have first been subjected to a process of gradual adaptation by repeated subculture over many weeks. It was found that strain C27 grew well without previous adaptation on agar plates and in broth enriched with rabbit serum when cholesterol (0·1 mg./ml.) was also added. This finding proved useful when rabbit serum broth cultures were required for injecting rabbits to prepare antisera (Edward & Fitzgerald, 1951).

The concentrations of cholesterol and AIL necessary for growth

The least amount of cholesterol necessary to give good growth was determined using two different concentrations of AIL, 4 and 0·4 mg./ml. All the strains grew well with 0·1 mg. cholesterol/ml.; with 0·03 mg./ml. growth was not so good (Table 2). Two strains, C27 and C55, grew better with 0·4 mg. AIL/ml. than with 4 mg./ml.; with the larger amount of AIL a higher concentration of cholesterol was required. Strain M46 did not grow on the media containing 0·4 mg. AIL/ml. Using the higher concentration of AIL good growth of this strain was obtained with 0·1 mg. cholesterol/ml.; with twice this amount of cholesterol growth was less good.

Table 2. *Effects of varying amounts of cholesterol in media containing two different concentrations of acetone-insoluble lipid (AIL) 4·0 or 0·4 mg./ml.*

Concentration of cholesterol (mg./ml.)	Strain C27		Strain L4		Strain B2		Strain M46		Strain C55	
	4	0·4	4	0·4	4	0·4	4	0·4	4	0·4
0·5	+	+	+	±	+	+	±	–	+	+
0·2	±	+	+	+	+	+	±	–	±	+
0·1	±	+	+	+	+	+	+	–	tr.	+
0·03	–	±	+	+	tr.	tr.	–	–	–	±
0·01	–	–	tr.	±	–	–	–	–	–	–

+ represents good growth. ± represents poor growth. tr. represents a trace of growth only. – represents no growth.

In a later experiment, in which 0·8 mg. of another preparation of AIL/ml. was incorporated in the medium, 0·01 mg. cholesterol/ml. was found to be the least amount giving good growth of strain C27. Different suspensions of cholesterol were used in the two experiments, and the difference in the results may possibly have been due to differences in the amount of cholesterol in a finely dispersed state. It was not always possible to obtain suspensions of the same quality. In two other experiments 0·01 mg. cholesterol/ml. was the least amount giving good growth, when incorporated in the medium with bovine albumin (5 mg./ml.).

Varying amounts of AIL were added to media containing 0·1 mg. cholesterol/ml. Strains C27, L4 and C55 grew with each of the concentrations of AIL tested, from 2 to 0·2 mg./ml. (Table 3). Strains B2 and H17 only grew with the largest amount (2 mg./ml.).

Table 3. *Effect of varying amounts of acetone-insoluble lipid (AIL) in media containing* 0·1 *mg. cholesterol/ml.*

Added AIL (mg./ml.)	Strain C27	Strain L4	Strain B2	Strain H17	Strain C55
2	+	+	+	+	+
0·8	+	+	±	−	+
0·4	+	+	−	−	+
0·2	+	+	−	−	+

Symbols as in Table 2.

These experiments suggested that optimum growth was obtained when there was a balance between the proportions of cholesterol and AIL in the medium. Strains appeared to vary in the amount of AIL which was necessary for their growth. There was also a suggestion from these and other experiments that growth was inhibited or impaired when cholesterol or other lipid fractions were added in amounts greater than those giving optimal growth.

Attempts to identify the factor in AIL assisting growth

Although growth of most strains was obtained on media containing cholesterol and bovine albumin, results were not consistent and growth appeared comparatively slowly. On media in which cholesterol was incorporated with AIL, consistently good growth equal to that on media enriched with horse serum was obtained with all except two strains. Therefore attempts were made to identify the material in AIL which was responsible for full development of growth.

The insoluble fraction obtained from egg-yolk by precipitation with acetone (AIL) was purified by reprecipitation three times with acetone; it still gave good growth in media containing cholesterol. A preparation of AIL was fractionated by precipitating a chloroform solution with ethanol. The precipitate (kephalin fraction), tested for activity without further purification, gave as good growth on media containing cholesterol as did the original AIL. The lecithin fraction, obtained by precipitating the alcohol-soluble portion with acetone, gave only a moderate growth which was slow in appearing. Purified preparations of lecithin and kephalin, prepared by Dr S. Wilkinson of the Chemical Division, The Wellcome Research Laboratories, when incorporated in the medium with cholesterol, gave only moderate and slow growth. There was no growth when either preparation was added to the medium without cholesterol. Similar results were obtained with two other purified preparations of lecithin and kephalin. The other fractions obtained during the process of purification were also tested for activity. Activity did not seem to reside in any one particular fraction, but it appeared from this and other experiments that, once a separation of AIL into lecithin and kephalin fractions had been made by ethanol precipitation, activity was lost during the subsequent repeated steps of acetone precipitation used in purification. An impure preparation of sphingomyelin was inactive.

Attention must be drawn to certain technical difficulties encountered in this part of the investigation. In order to determine the presence of growth and to assess its development accurately, it was necessary to observe cultures with a dissecting microscope, using transmitted light. This was only possible when the medium was relatively translucent. Many of the lipid fractions tested were difficult to incorporate in the medium as finely dispersed suspensions. The media were often opaque, contained numerous oily globules and had an uneven surface. Under these conditions it was difficult to assess the amount of growth.

It was concluded that in media containing cholesterol the acetone-insoluble material from egg-yolk assisted growth, so that it was as good as on serum media and was better than on media containing cholesterol + albumin. Lecithin and kephalin fractions, purified by acetone-precipitation, did not give such good results, growth being no better than with cholesterol and albumin.

Effect of cholesterol esters and of other sterols

On media containing albumin or AIL equally good growth was obtained with three different preparations of cholesterol. One preparation had a purity exceeding 99 %. It must, however, be pointed out that there are always present in aqueous suspensions of cholesterol hydroxylated and oxygenated derivatives, such as 7-hydroxycholesterol (Haslewood, 1944). Strains C 27 and L 4 grew moderately well on media containing starch and cholesteryl hydrogen phthalate (0·05 mg./ml.), an ester which is slightly soluble in water; there was no growth when this ester was added to the medium without starch. No growth was obtained with cholesteryl acetate, cholesteryl stearate or cholesteryl oleate, tested at concentrations of 0·3, 0·1 and 0·03 mg./ml., when added to the medium alone, or with starch, bovine albumin or AIL.

Four other sterols, very kindly provided by Prof. G. A. D. Haslewood, were tested for ability to promote growth. Growth, equal to that given by cholesterol, was obtained with cholestanol or stigmasterol incorporated in the medium with bovine albumin or AIL; the minimum active concentrations were approximately the same as for cholesterol. Under the same conditions no growth was obtained with ergosterol and coprosterol, but this may have been due to a failure to obtain suitably dispersed suspensions. When an aqueous suspension of coprosterol was added to the medium it appeared to flocculate and crystallize out. The technique, which had been used for the other sterols, was not successful for obtaining a finely dispersed suspension of ergosterol.

Effect of protein and lipid fractions of serum in promoting growth

Since growth was obtained by adding ether-soluble fractions of egg-yolk to the medium, it seemed possible that the ability of serum to promote growth might be due to its lipid constituents. The method of Hartley (1925) was used to obtain a preparation of serum protein freed from lipids. When this was added to the basal medium alone there was no growth of the strain tested. There was also no growth on a medium in which were incorporated the lipids extracted from the serum in the preparation of the lipid-free protein. However, when the lipids and the lipid-free protein were added together there was a moderately good growth of strain C 27. Some growth was obtained by adding to the medium a mixture of serum lipids and AIL (5 mg./ml.), but there was no growth on media to which had been added cholesterol (0·1 mg./ml.) and serum lipids. Good growth occurred on media in which the serum lipids had been incorporated together with bovine albumin. These findings suggest that both protein and lipid fractions of serum are concerned in its ability to promote growth of organisms of the pleuropneumonia group.

Equally good growth was obtained on media containing cholesterol and a preparation of serum proteins freed from lipids by Hartley's method, as on media containing cholesterol and bovine albumin. The effect of this fraction of bovine albumin would thus appear to be due to the protein itself and not to any lipids possibly associated with it.

Inhibition by oleic acid

Recently considerable attention has been paid to the effects of fatty acids, particularly unsaturated acids such as oleic acid, on the growth of bacteria. Excessive concentrations have been found to be inhibitory, some organisms being particularly sensitive, whereas for other organisms small amounts of certain fatty acids are essential for growth (Pollock, 1949). Since the growth of pleuropneumonia-like organisms on media containing cholesterol was favoured by substances, such as starch and albumin, which bind fatty acids, the action of oleic acid on organisms of the pleuropneumonia group was studied. When varying amounts of oleic acid were added to media enriched with 5 % horse serum 0·1 mg. oleic acid/ml. inhibited all strains, though normal growth occurred with half that amount. In media containing 2·5 % horse serum 0·05 mg. oleic acid/ml. was inhibitory. Amounts of oleic acid exceeding 0·05 mg./ml. were also inhibitory in media containing cholesterol and bovine albumin instead of serum.

Growth of *A. bovis* and strain C48 on media containing 5 % horse serum appeared in two experiments to be improved by adding small non-inhibitory amounts of oleic acid. This was not confirmed in subsequent experiments, and it was not possible to obtain regularly good growth of these strains on media containing cholesterol and albumin, or cholesterol and AIL by adding varying amounts of oleic acid. The addition of Tween 80 did not improve growth on media containing cholesterol.

DISCUSSION

The evidence presented here suggests that cholesterol is an essential medium component for growing certain organisms of the pleuropneumonia group isolated from animals. Growth was obtained on a basal medium to which cholesterol had been added as a finely dispersed suspension. For satisfactory growth it was necessary to incorporate also in the medium starch, bovine albumin or the acetone-insoluble fraction of egg-yolk (AIL), previously described. No growth resulted from the addition to the medium of any of these latter substances, alone or in combination, in the absence of cholesterol. For maximal growth enough cholesterol had to be added to give a concentration of at least 0·01 mg./ml. When horse serum was used for enrichment, 2·5 % (v/v) was the least concentration giving good growth. Mayer & Schaeffer (quoted by Bloor, 1943) found horse serum to contain 0·83 mg. cholesterol/ml. About 70 % of this cholesterol was present as esters of fatty acids (Bloor, 1943). Thus the addition of 2·5 % horse serum to the medium could provide enough free cholesterol to give a concentration of *c.* 0·006 mg./ml., which is in close agreement with the concentration found to be required when a suspension of cholesterol was used.

Cholesterol has been found necessary for the growth and development of certain insects; for instance, McKinnis (1947) showed that cholesterol or another sterol was required for the development of larvae of the black carpet beetle. Cailleau (1937), in a study of the growth requirements of *Trichomonas*, found cholesterol to be essential for the growth of *T. columbae*; certain other sterols and the esters of cholesterol were also effective. Griffin & McCartin (1949) showed that cholesterol or its esters with oleic and palmitic acids could replace serum in media used for growing *Entamoeba histolytica*; a concentration of 0·09 mg./ml. was effective. Certain bacteria, such as soil bacteria of the genus *Proactinomyces* (Turfitt, 1947) and *Mycobacterium smegmatis* (Sobel & Plaut, 1949) have been shown to oxidize and decompose cholesterol, growth taking place when cholesterol was the sole source of carbon. Lwoff (1947) investigated the factor in serum which permitted growth of *Moraxella lacunata* and *Neisseria gonorrhoeae*. The organisms grew poorly, or not at all, in plain broth, unless it was enriched with serum. Good growth followed the addition to the broth of a soluble ester of cholesterol instead of serum, but equally good growth was obtained simply by diluting the broth. Lwoff concluded that cholesterol was not a growth factor in the strict sense; it acted by neutralizing inhibitory substances in the broth, which were probably fatty acids. Thus, although cholesterol is essential for the growth of certain insects and Protozoa, it is not known to be an essential nutrient for any bacteria.

The importance of cholesterol in the cultivation of pleuropneumonia-like organisms was first suggested by the observations of Partridge & Klieneberger (1941). They showed that oily globules, found in colonies of the L1 organism, consisted largely of free cholesterol. The amount of cholesterol, associated with the organisms in the culture, was comparatively large; the total lipid which could be extracted from the organisms was about 40 % of their dry weight and about 40 % of this was cholesterol. It did not appear that this cholesterol was synthesized by the organism, for there was shown to be a decrease of cholesterol in the medium during growth. The authors failed to obtain growth by adding cholesterol or cholesteryl palmitate instead of serum to the medium. The L1 organism has not yet been included among the strains which we have tested on media containing cholesterol and albumin, or cholesterol and AIL. Observations on the growth requirements of this organism will be of interest, for it is typical of those strains which are the pleuropneumonia-like forms of bacteria. A comparison of the growth requirements of the corresponding bacillary and pleuropneumonia-like forms may assist in elucidating the relationship of the one to the other. It is to be noted that all pleuropneumonia-like forms which have been isolated from Gram-negative bacilli have needed media enriched with serum, even though the bacillary form did not; on the other hand, pleuropneumonia-like forms, isolated from Gram-positive bacilli, have not needed serum (Dienes, 1950).

Two other compounds, cholestanol and stigmasterol, were as effective as cholesterol in promoting growth, while ergosterol and coprosterol appeared to be inactive (although the tests of these latter compounds may have been unsatisfactory). Examination of a larger number of sterols would be necessary before an attempt could be made to correlate activity with chemical structure. Cholesterol esters of acetic, stearic and oleic acids were inactive. Esterification, however, did not necessarily abolish the activity of cholesterol, because growth was obtained with cholesteryl hydrogen phthalate. The action of this ester may have been facilitated by its water solubility.

Growth could seldom be obtained by adding cholesterol alone to the basal medium; it was necessary to incorporate with it certain substances, namely starch, bovine albumin or AIL. It seems likely that their action, at least in part, was to bind fatty acids so that they were not present free in the medium in toxic amounts; oleic acid in excessive concentrations was shown to be inhibitory. Cholesterol itself forms complexes with fatty acids. However, this ability cannot explain its action in promoting growth, because it could not be replaced by other substances such as bovine albumin, which neutralize the inhibitory effects of fatty acids.

Of the eight strains tested two, namely strain C48 and the strain of *Asterococcus bovis*, could not be grown regularly even with cholesterol and AIL. In the early experiments there appeared to be something in AIL which was inhibitory for strain C48. Later, after a further period of subculture, there was some growth of this strain on media containing cholesterol and AIL. Growth of *A. bovis* was erratic; it grew in some experiments but not in others on media with cholesterol and albumin or cholesterol and AIL. Also, unlike the other strains it did not grow well on media enriched with egg-yolk instead of serum. Thus strains appeared to vary in the ease with which they could be grown; of those studied *A. bovis* and strain C48 were the most exacting in their requirements. It would seem that the conditions for the growth of organisms of this group are highly complex and that all the factors necessary have not yet been elucidated.

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An Organism Able to Metabolize 3-Methylglucose and 3-Methylfructose

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SUMMARY: An organism was isolated from soil, which grew in a basal mineral medium with either 3-methylglucose or 3-methylfructose as sole source of carbon. The bacterium is a member of the *coli-aerogenes* group, intermediate type 1. Grown in presence of either methyl-sugar, under suitable conditions, washed suspensions of the organism oxidize either 3-methylglucose or 3-methylfructose. Under anaerobic conditions, the two methyl-sugars are broken down with the formation of hydrogen, carbon dioxide and acid. The ability to attack 3-methylglucose or 3-methylfructose was acquired by growth in presence of either of the two methyl-sugars but not in the presence of glucose.

When 3-methylglucose is fed or administered to rats it is largely excreted in the urine unchanged (Campbell, 1949). The present investigation was undertaken in an attempt to discover whether micro-organisms exist which are able to attack and utilize both 3-methylglucose and 3-methylfructose, and whose existence might affect the use of these sugars in feeding experiments.

METHODS

Growth media. A basal mineral medium was prepared as follows: $(NH_4)_2HPO_4.2H_2O$, 4·0 g.; KH_2PO_4, 0·4 g.; NaCl, 1·0 g.; $MgSO_4.7H_2O$, 0·7 g.; $FeSO_4.7H_2O$, 0·3 g.; distilled water to 1·0 l.; pH adjusted to 7·0. The basal medium was distributed in 9 ml. portions in miniature Roux bottles and sterilized by autoclaving (110°). Solutions (10 %, w/v) of 3-methylglucose or 3-methylfructose were sterilized by filtration through Seitz filters. One ml. of one or other of these solutions was added to each Roux bottle; for anaerobic growth the medium was distributed in flasks.

Preparation of washed suspensions of bacteria. After 36 hr. incubation at 30° cultures were centrifuged; the cells were washed once in distilled water and made up into a suspension in water. The dry weight of cells in such suspensions was determined on the Hilger Spekker absorptiometer previously calibrated by dry-weight determinations.

Respiration experiments. Oxygen consumption was measured in Warburg manometers containing washed cell suspension, phosphate buffer (pH 7·0) and 3-methylglucose or 3-methylfructose; NaOH solution and filter-papers were placed into the centre cups to absorb CO_2. The production of CO_2 was determined in parallel experiments by omitting NaOH from the centre cup and releasing retained CO_2 at the end of the experiment by tipping H_2SO_4 from a second side-bulb. Rates of oxidation are expressed as Q_{O_2}.

Fermentation experiments. The anaerobic breakdown of the methyl-sugars was studied in Warburg manometers. Gas production was measured in manometers filled as above but containing nitrogen gas; CO_2 retained was measured by the acid-tip method as above; manometers were set up in duplicate, one of each pair containing NaOH in the centre cup to absorb CO_2 if produced. Acid and gas production were measured in manometers containing washed suspension, $NaHCO_3$ and 3-methylglucose or 3-methylfructose; the manometers were filled with a gas mixture containing 95 % (v/v) N_2 and 5 % CO_2. Acid production was calculated from the decrease in $NaHCO_3$ concentration determined after the experiment by the usual acid-tip method. Gas production in the bicarbonate buffer system is expressed in terms of CO_2.

Isolation of organism utilizing methyl-sugars

Several bottles each of the two media containing the respective methyl-sugars were inoculated with garden soil and aerobically incubated at 25°. In all cases the medium became turbid within 24–48 hr. During the first series of experiments, four serial subcultures were made into fresh medium at 48 hr. intervals. In a second series of experiments, four subcultivations were made at 24 hr. intervals. At the end of these periods the cultures were plated on the two media solidified with 3 % of agar.

No consistent growth was obtained in the experiments using 3-methylglucose, the fourth subcultivation giving a slight amount of growth which proved to consist of a mixture of many different organisms. These experiments were discontinued, since an organism utilizing 3-methylglucose was isolated from the cultures containing 3-methylfructose.

The cultures grown with 3-methylfructose grew heavily and proved to consist of a mixture of four or five morphologically distinct organisms. In the culture series which had been subcultivated at 48 hr. intervals the predominant organism was isolated in pure culture and identified as a species of *Pseudomonas*. This organism, however, proved unable to grow in liquid medium with 3-methylfructose as sole source of carbon. This *Pseudomonas* species was not predominant on plates of cultures subcultivated at 24 hr. intervals, and four different colonies were selected from the plating of this series for further investigation. Organisms from these colonies were grown in 3-methylfructose media and then replated until pure cultures were obtained. At the same time the organisms were inoculated into Roux bottles containing 150 ml. 3-methylfructose medium, incubated at 30° for 36 hr., harvested on the centrifuge, washed once in distilled water, resuspended in water and tested for ability to oxidize 3-methylfructose. Growth was poor with one strain (not further studied) but heavy with one and moderate with the remaining two. The respiration experiments indicated that the three organisms oxidized 3-methylfructose at different rates. The organism which gave heavy growth in 36 hr. at 30° had Q_{O_2} (3-methylfructose) $= 12$–24 in preliminary experiments; this organism was used for the studies reported in this paper.

Identification of organism

The organism was identified as *Bacterium coli* intermediate type 1 (Wilson, Twigg, Wright, Hendry, Cowell & Maier, 1935). The bacterium has the morphological and cultural characteristics of *Bact. coli* intermediate type 1, with an optimal growth temperature range of 25–30°. This organism belongs to the type which is one of the commonest of all coliform bacteria found in soils of this country (Bardsley, 1934). The species with closest resemblance to our organism listed in *Bergey's Manual* (1948) is *Escherichia freundii* (Braak) Yale.

The biochemical characteristics of our strain can be summarized as follows: acid and gas formation, under aerobic conditions, took place in peptone broth after 18 hr. with arabinose, glucose (also at 37°), 3-methylglucose, fructose, 3-methylfructose, galactose, mannitol, sucrose, maltose and cellobiose. Acid and traces of gas after 3 days with rhamnose and lactose; acid but no gas with glycerol after 40 hr.; neither acid nor gas from dulcitol, raffinose, inulin or starch. Under anaerobic conditions (N_2/CO_2 or H_2/CO_2) acid and gas in peptone broth or Stephenson's medium with glucose, fructose, 3-methylglucose or 3-methylfructose (Stephenson, 1949).

Other biochemical reactions: nitrite formed from nitrate but no N_2 liberated; hydrogen sulphide positive; indole negative; methyl-red positive; Voges-Proskauer negative; citrate utilization positive; urease negative; catalase positive; gelatine liquefaction negative after 14 days' incubation.

Metabolism of 3-methylfructose and 3-methylglucose

The growth tests indicated that the organism could utilize either 3-methylfructose or 3-methylglucose for aerobic or anaerobic growth. The organism was therefore grown on a larger scale for metabolic investigations in the defined basal medium containing the appropriate methyl-sugar. The ability of washed suspensions to oxidize the methyl-sugars was first tested and experiments were

Table 1(*a*). *Metabolism of 3-methylfructose by washed suspensions of cells grown in the presence of 3-methylfructose*

(Organism grown for 36 hr. at 30° in basal medium + 0·5 % (w/v) 3-methylfructose. Suspension density: 22 mg. dry-weight cells/ml. For oxygen consumption: main compartment contained 1·0 ml. 0·1 M phosphate buffer (pH 7·0) and 1·0 ml. cell suspension; 0·5 ml. 0·01 M 3-methylfructose (or water in controls) in side bulb, and 0·2 ml. 10 % NaOH with filter-papers in inner cup. For CO_2 production, conditions as above and in addition 0·5 ml. 2 N-H_2SO_4 in side bulb 2 but without NaOH in inner cup; air.

For fermentation experiments: main compartment 1·0 ml. washed suspension + 1·0 ml. 0·03 M-$NaHCO_3$; 0·5 ml. 0·01 M 3-methylfructose in side bulb 1, and 0·5 ml. 2 N-H_2SO_4 in side bulb 2; 95 % N_2 + 5 % CO_2; all values in μl.)

Atmosphere		Cells + substrate (μl.)	Cells alone (μl.)	Difference (substrate) (μl.)
Air	Q_{O_2}:	12·9	1·8	11·1
	Oxygen consumption (completion)*:	790	363	427
Air	Q_{O_2}:	23·6	4·6	19·0
	Oxygen consumption (completion)*:	699	206	493
	CO_2 production (completion)*:	695	245	450
95 % N_2 + 5 % CO_2	Gas production, as CO_2:	307	24	283
	Acid production, as CO_2:	208	0	208

* Theory for complete oxidation = 840 μl.

Table 1(*b*). *Metabolism of* 3-*methylglucose by washed suspensions of cells grown in presence of* 3-*methylglucose*

(Conditions similar to Table 1(*a*) with 3-methylglucose substituted for 3-methylfructose.)

Atmosphere		Cells + substrate (μl.)	Cells alone (μl.)	Difference (substrate) (μl.)
Air	Q_{O_2}:	24·5	8·7	15·8
	Oxygen consumption*			
	150 min.:	624	299	325
	Completion, 12 hr.:	1184	728	456
Nitrogen	Hydrogen production:	227	17	210
	CO_2 production:	233	31	202
	Q_{H_2}:	7	0	7
95 % N_2+5 % CO_2	Gas production, as CO_2:	399	16	383
	Acid production, as CO_2:	159	0	159

* Theory for complete oxidation = 840 μl.

continued until the rate of oxygen consumption in presence of the given methyl-sugar had fallen to the rate in manometers without substrate. Table 1, *a* and *b*, shows that for both methyl-sugars the oxidation continued until the oxygen uptake (allowing for blank respiration) had reached a value approximately 55 % of that required theoretically for complete oxidation of substrate. The CO_2 output was approximately equal to the oxygen consumption. When washed suspensions were incubated anaerobically in N_2 or N_2+CO_2 atmospheres, evolution of gas took place. With 3-methylglucose in N_2, the gas evolved consisted of approximately equal volumes of hydrogen and carbon dioxide. Experiments in bicarbonate buffer indicated (Table 1*a* and *b*) that, under anaerobic conditions, acid was also produced. Allowing for the fact that the gas produced contained hydrogen, the acid formed was approximately equivalent to the CO_2 formed during the fermentation.

Effect of presence of substrate during growth

The organism was grown in basal medium with 3-methylfructose, 3-methylglucose or glucose as sole carbon source. Washed suspensions were prepared and the ability to oxidize the methyl-sugars tested. Table 2 shows that neither

Table 2. *Effect of growth substrate on ability to oxidize* 3-*methylglucose and* 3-*methylfructose*

(Conditions similar to those given in Table 1(*a*). Rate of oxygen uptake studied over 1 hr. and expressed as Q_{O_2} = μl. oxygen taken up/hr./mg. dry-weight cells. Figures in ( ) are those for endogenous respiration blanks.)

	Respiration substrate		
Carbon source in growth medium	Glucose Q_{O_2} (μl.)	3-Methylglucose Q_{O_2} (μl.)	3-Methylfructose Q_{O_2} (μl.)
Glucose	32·5 (0·5)	1·0 (0·5)	0·0 (0·5)
3-Methylglucose	—	73·6 (9·5)	65·8 (9·5)
3-Methylfructose	—	69·2 (13·2)	64·5 (13·2)

3-methylfructose nor 3-methylglucose was attacked by cells grown in glucose but that both methyl-sugars were attacked by cells grown in the presence of either. It appears probable that the primary breakdown of the methyl-sugars requires the presence of an adaptive enzyme evoked by the presence of either 3-methylglucose or 3-methylfructose.

The authors are indebted to Prof. F. G. Young, F. R. S., for suggesting this research and for the supply of 3-methylglucose and 3-methylfructose (from Messrs Ayerst, McKenna and Harrison Ltd., Montreal) and to Dr E. F. Gale for his valuable help throughout this investigation.

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The Production of Swarmers in *Rhizobium* spp.

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SUMMARY: In certain strains of *Rhizobium* spp., tiny spherical swarmers, with a central nucleus and one or occasionally two flagella, are released from the cell lumen of specialized large bacilli, the 'barred' forms, of which the 'bars' are modified cross-walls. The swarmers exhibit appearances suggestive of conjugation.

In other strains true swarmers are not produced; their function is performed by small motile cells produced by normal cell division.

The outline of the life cycle of *Rhizobium* spp. is well known, but some controversy exists upon the mode of formation of the small swarmer elements. The view of Thornton & Gangulee (1926) and others, that these arise by the break-up of the 'barred' rods, of which each stainable band represents a single swarmer cell, appears to be widely accepted; that of Lewis (1938), that they arise by the continued process of cell division, probably refers, as will be shown, to the production of small, motile elements in strains which do not form true swarmers, whereas the latter originate within the barred bacilli and are released by the rupture of the cell wall. This type of reproduction is found, in a variety of comparable forms, in many bacterial groups (Bisset, 1950).

METHODS

Thirty-two strains of *Rhizobium* spp. were examined. Twenty-seven were newly isolated from root-nodules of cultivated peas and beans, everlasting sweet-pea, wild vetch, lupin and various species of clover. Five were laboratory cultures.

Initial cultures were made by sterilizing the surface of the root-nodules with 0·1% mercuric chloride, crushing them between sterile slides, and inoculating upon nutrient agar containing 0·3 % Yeastrel.

Smears were stained by Gram's method, by acid-Giemsa for nuclear structures, by tannic acid violet for cell walls (see Bisset, 1950), and by silver impregnation for flagella. Flagella were also demonstrated by electron microscopy.

OBSERVATIONS

General morphology. At most stages of culture the majority of strains consisted of small unicellular bacteria (Pl. 2, fig. 16), either motile or non-motile, but some young cultures, especially those from lupin and everlasting sweet-pea, contained large, septate bacilli, with from four to ten or more cells (Pl. 1, fig. 4; Pl. 2, figs. 11, 12). The morphology of the bacteroids found in the root-nodules will be described in a separate study.

Flagella. Bacteroids and other stages within the plant were devoid of flagella. The large bacilli were peritrichously flagellate (Pl. 1, fig. 6). The swarmers had a single flagellum or, less frequently, two flagella (Pl. 2, figs. 8–10 and 14). The small motile bacteria, in strains which did not produce swarmers, had four or five flagella arranged in a rather haphazard manner, polar, peritrichous or in a tuft at one side of the cell (Pl. 2, fig. 16).

Production of swarmers. In strains which did not produce true swarmers, many cells in the motile stage were so small as to resemble them, but were much less regular in their morphology. They appeared to be formed by the normal process of cell division from larger cells, and a complete series of intermediate stages could often be seen in a single culture. This condition was typical of strains from cultivated peas, beans and clovers, although true swarmers may occasionally have been produced by some of these.

In the strains from lupins, sweet-peas and vetches the swarmers were produced from barred bacilli of the kind so frequently described in the past (Pl. 1, figs. 1, 2). These occurred in cultures of the large multicellular bacilli (Pl. 1, fig. 4), which they resembled in most respects, although the specialized reproductive bacilli were slightly larger than the vegetative bacilli (cf. Pl. 1, figs. 1, 4). The barred appearance was due to an aggregation of stainable material upon the interior cross-walls of the bacilli. The tiny coccoid swarmers were produced from the lumen of these cells and emerged by the rupture of the cell wall (Pl. 1, figs. 1, 2; Pl. 2, figs. 11, 12). It is reasonable to assume that this stainable material is concerned with the formation of the cell constituents of the swarmers. By electron microscopy this appearance of a thickened cell wall surrounding a hollow lumen was occasionally seen (Pl. 1, fig. 3). In both electron and photomicrographs these mother cells appeared as though softened and, eventually, empty and disrupted (Pl. 1, figs. 1, 6 and 7; Pl. 2, figs. 11, 12). In nearly all micrographs swarmers were seen attached to, or emerging through, the cell wall of the mother bacillus (Pl. 1, figs. 5–7; Pl. 2, fig. 8).

The swarmers frequently appeared in arrangements suggestive of conjugation (Pl. 1, fig. 1; Pl. 2, figs. 13, 14). The alternative explanation, that these cells were dividing instead of fusing, is not in accordance with the configuration of the nuclear elements and of the flagella; the former being more closely fused than the cytoplasm, whereas the division of the nucleus normally precedes that of the remainder of the cell; the latter being equally well developed upon both cells. In fact the swarmers, as such, did not appear to reproduce, but rapidly assumed the form of tiny bacilli, each with from one to three flagella (Pl. 2, figs. 11, 12 and 15), which grew up to form a new generation of vegetative bacteria, or which, alternatively, were capable of infecting the root-hairs of susceptible plants.

DISCUSSION

The appearances seen in both electron and photomicrographs of the production of swarmers in *Rhizobium* spp. do not support either of the accepted theories of the mechanism of this process. It is not true that each stainable band upon the barred bacilli represents a single swarmer, nor that these bacilli fragment to produce the swarmers. They are released from the relatively lightly stained cell lumen between the bands, which appear to be modified secretory cell walls (Fig. 1).

It is true that small motile cells are produced by the normal processes of cell division in certain strains of *Rhizobium* spp., but these are not swarmers, although they may perform the same function.

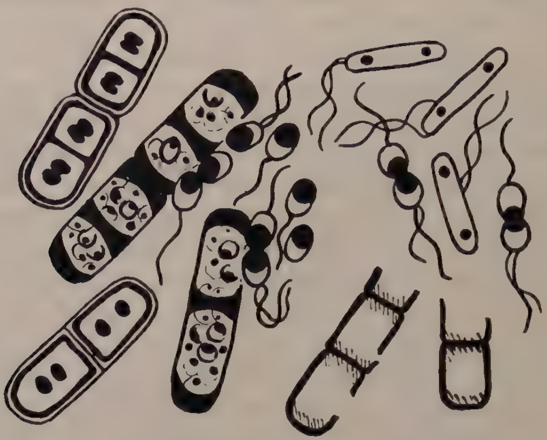

Fig. 1. Production of swarmers from the cell lumen of the large 'barred' bacilli. The bars are specialized cross-walls, and the swarmers are released by rupture of the cell wall. They show signs of conjugation and are rapidly transformed into small rods.

The independent occurrence of these two methods of producing small motile cells serves to clarify the divergent theories which have been suggested to explain the appearance of swarmers. It is especially significant that true swarmers are less readily produced by the strains of bacteria found in association with the best-known and most important leguminous crops.

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EXPLANATION OF PLATES

(All figures except Pl. 2, fig. 16, are of a strain of *Rhizobium* derived from lupin. Figs. 1, 2, 4, 11 and 12 are photomicrographs, the remainder electron micrographs.)

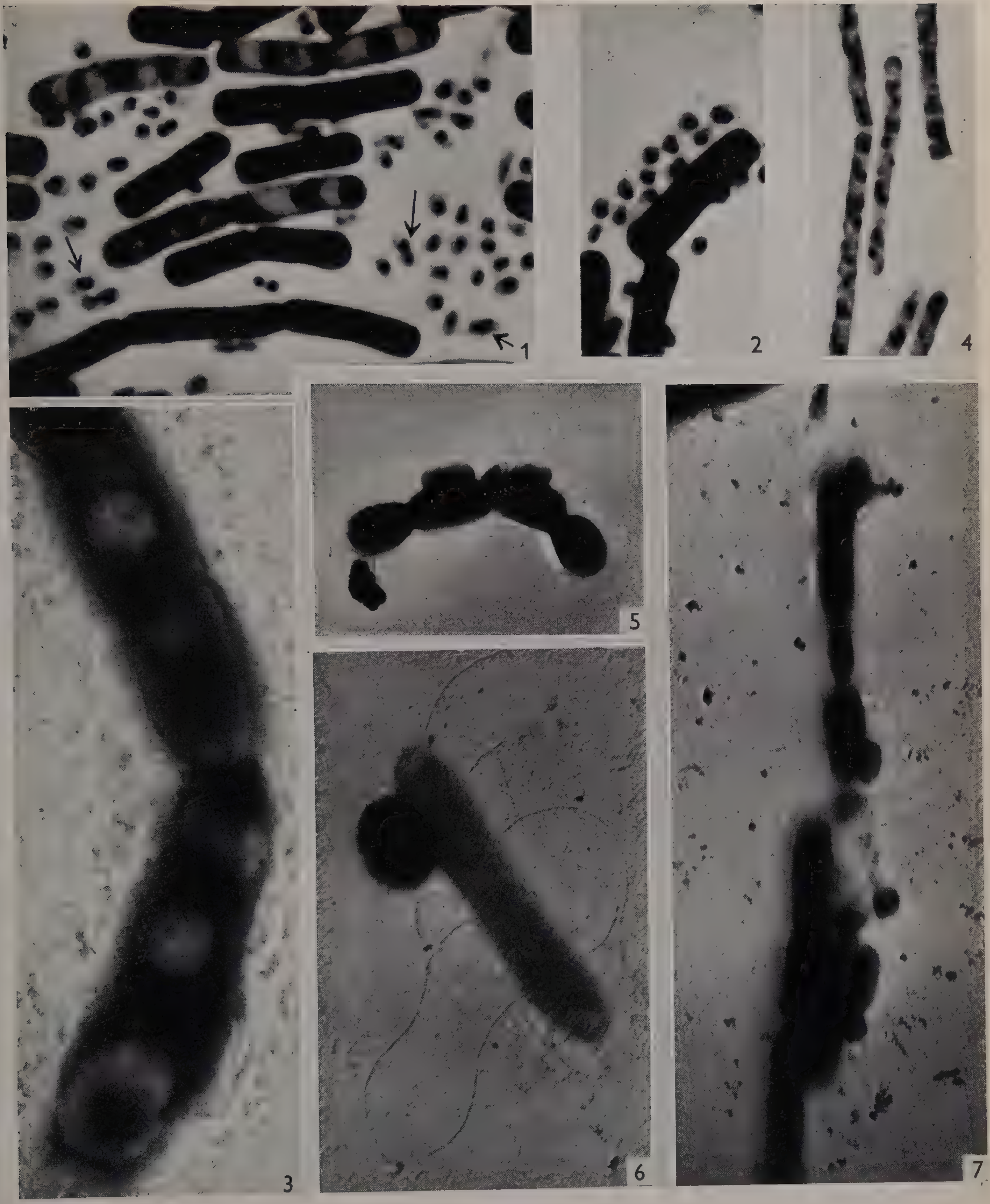
Plate 1

Figs. 1, 2. Release of swarmers from large 'barred' bacilli. The bars are obviously modified cross-walls. The arrows indicate the apparent fusion of swarmers. In one bacillus the outer wall is softened and disrupted. Acid-Giemsa. × 3000.

Fig. 3. Barred bacillus by electron microscopy. Here also the identity of the bars with cross-walls is apparent. × 16,000.

Fig. 4. Large multicellular vegetative bacilli. Acid-Giemsa. × 3000.

Figs. 5–7. Release of swarmers. In fig. 6 the flagella of the bacillus are seen. It is soft and electron transparent (× 10,000). In figs. 5 and 7 collapsed and disrupted bacilli are seen (× 50,000). In all three swarmers are attached to, or emerging through, the walls of the bacilli.



Figs. 1–7

K. A. Bisset and C. M. F. Hale—Swarming in *Rhizobium*. Plate 1

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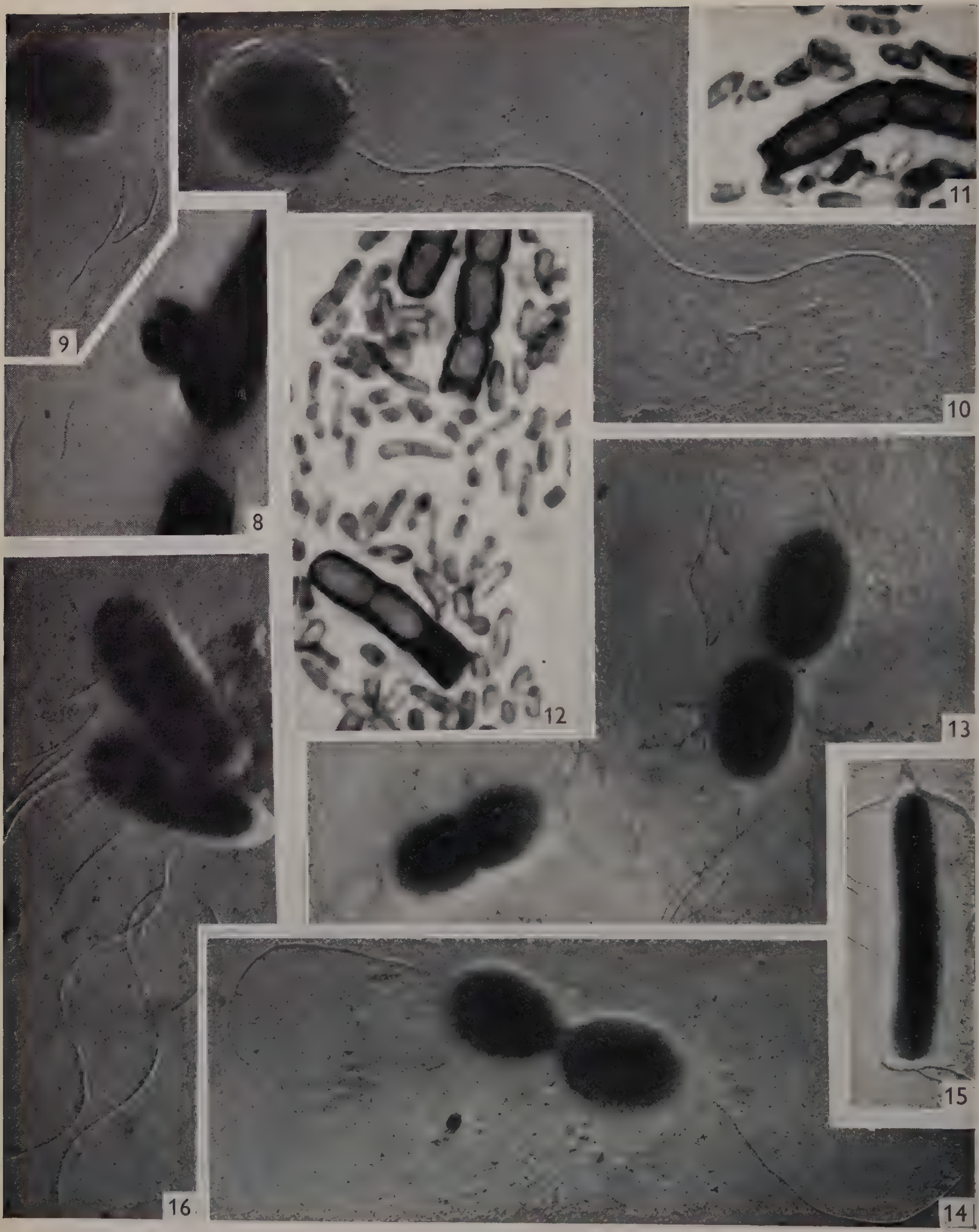

Figs. 8–16

K. A. BISSET AND C. M. F. HALE—SWARMING IN *RHIZOBIUM*. PLATE 2

PLATE 2

Figs. 8, 9. Swarmers, each with two flagella. × 10,000 and × 16,000 respectively.

Fig. 10. Swarmer with single flagellum. × 20,000.

Figs. 11, 12. Broken cell walls of bacilli, showing cross-walls; swarmers mainly transformed into small rods. Tannic-acid-violet. × 3000.

Figs. 13, 14. Swarmers apparently in various stages of fusion. × 16,000.

Fig. 15. Swarmer transformed into small rod. × 16,000.

Fig. 16. *Rhizobium* from small, white clover. Small cells, actively motile by lateral tufts of flagella. × 16,000.

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The Segregation of Streptomycin-Resistant Variants of *Mycobacterium tuberculosis* into Groups with Characteristic Levels of Resistance

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SUMMARY: Three initially streptomycin-sensitive strains of *Mycobacterium tuberculosis*, two from patients with tuberculosis, and one the laboratory strain H37Rv, were grown for 6 weeks on Herrold's agar medium containing graded concentrations of streptomycin. Discrete colonies of *M. tuberculosis* that grew on the streptomycin media were tested for streptomycin sensitivity in Tween 80 albumin medium.

In all three strains the distribution of resistance was discontinuous, the subcultures from the colonies being either sensitive, or resistant to streptomycin in three distinct ranges of minimal inhibitory concentrations, namely, 0·25–0·5, 1–2 and 32–64 μg./ml.; and of initial populations of $0{\cdot}4$–$1{\cdot}0 \times 10^9$ bacilli, about 1000 were in the first, slightly resistant group, about 100 in the second, and about 2 were in the third, highly resistant group.

Of the two patients from whom the two of the strains were isolated, one developed a highly resistant strain in his sputum during treatment, whereas the other developed a strain only of slight resistance, in spite of prolonged streptomycin treatment.

The occurrence of streptomycin-resistant variants of *M. tuberculosis* in populations of organisms which have not previously been exposed to the antibiotic has been demonstrated in laboratory strains by Vennesland, Ebert & Bloch (1947) and Yegian & Vanderlinde (1948), and in strains obtained directly from patients by Pyle (1947). Yegian & Vanderlinde inoculated plates of a medium containing the detergent Tween 80 and streptomycin with large numbers of bacilli, and found that the number of resistant colonies that grew depended on the concentration of streptomycin. The number of colonies decreased markedly when the concentration was increased from 1 to 10 μg./ml. and then to 100 μg./ml. With the higher concentrations, such as 100 and 1000 μg./ml., there was no significant difference in the number of colonies. Strains derived from colonies growing in the presence of 100 μg./ml. were also capable of growth in 1000 μg./ml. One of these highly resistant variants was found in every 10^8–10^9 bacilli in the original inoculum, though the proportions varied considerably with different strains and in different experiments with the same strain. Strains derived from colonies growing in the presence of 1 μg./ml. contained a larger proportion of highly resistant variants than did the original parent population. Meads & Haslam (1949), investigating the occurrence of streptomycin-resistant variants of *Klebsiella pneumoniae*, found that the predominantly sensitive population contained two groups of variants, a small number of organisms slightly resistant to streptomycin and a much smaller number of highly resistant organisms. In their experiments slightly resistant strains contained the same proportion of highly resistant organisms as did the parent population, namely, 0·5–4·6 per 10^{10} organisms in the inoculum.

In a previous publication (Mitchison, 1949), sensitivity tests on 398 streptomycin-resistant strains of *M. tuberculosis*, isolated from patients during their treatment with the antibiotic, were classified according to the concentration of streptomycin which inhibited their growth. There were three distinct groups of strains, each with a characteristic degree of resistance. In the first group strains were inhibited by 1–2 μg./ml. and were about four times less sensitive than the standard sensitive strain H37Rv; in the second, by 32–64 μg./ml; in the third group strains grew in at least 100 μg./ml. If we accept the view that the occurrence of resistant strains in the sputa of patients receiving streptomycin is due to the selection of the variants present in small numbers in the pre-treatment population of bacilli, then there are two possible explanations of the occurrence of these groups. Either all degrees of streptomycin resistance are to be found among these variants and the occurrence of the groups among the strains isolated from the patients are due to selective factors in the environment such as the maximum or minimum levels of streptomycin in the lesions; or the variants themselves tend to occur in these groups before the selective action of the presence of streptomycin in the lesions begins. An attempt has been made in this paper to establish whether the distribution of streptomycin resistance among variants occurring in a normal sensitive population is in fact discontinuous and whether the groups of variants with different resistances correspond with these found among strains isolated from treated patients.

METHODS

Strains of Mycobacterium tuberculosis

Three strains of tubercle bacilli of human type were used:

(1) H37Rv, a virulent laboratory strain from which variants with a high degree of streptomycin resistance have been obtained.

(2) Pa, a strain isolated from the sputum of a patient with pulmonary tuberculosis before treatment with streptomycin and preserved by drying in gelatin-ascorbate by the method of Stamp (1947). The patient received 1 g. of streptomycin per day over a period of 6 months. Numerous cultures isolated from the sputum from the sixth week onwards yielded strains only two to four times less sensitive than the standard strain H37Rv.

(3) Pr, a strain isolated from a patient with a similar pulmonary condition before treatment was started with the same dosage of streptomycin over the same period of time. This culture was also preserved by drying. During treatment cultures from this patient yielded several strains capable of growth in 1000 μg. streptomycin/ml.

Streptomycin. The streptomycin used was the calcium chloride complex prepared by Merck and Co. (Rahway, N.J., U.S.A.).

Medium. Batches of Herrold's egg agar medium were prepared with the following composition: Lemco meat extract (Oxo Ltd., London), 3 g.; peptone, 10 g.; sodium chloride, 5 g.; glycerol, 50 ml.; agar, 15 g.; and distilled water to 1000 ml.

This base was filtered, adjusted to pH 7·3, and autoclaved at 15 lb./sq.in.

for 20 min. Before use it was melted, cooled to 55–60° and the yolk of an egg, separated with sterile precautions, was added to each 150 ml. The total amount of the medium required for each experiment was prepared in one batch. From this, appropriate quantities were transferred to a series of conical flasks containing streptomycin so that final streptomycin concentrations of 0, 1, 2, 4, ..., 1024 μg./ml. were obtained. The contents of each flask was distributed in 4 ml. lots to 3 × 1 in. flat-bottomed tubes plugged with cotton-wool and was allowed to set so that a flat plaque was left covering the bottom of the tube. The tubes were dried for about 4 hr. at 37°.

Table 1. *The design of the test for growth in the presence of streptomycin*

(The figures indicate the number of tubes of Herrold's medium inoculated with strains Pa.)

	Concentration of streptomycin in Herrold's medium (μg./ml.)											
	0	1	2	4	8	16	32	64	128	256	512	1024
Culture dilution	No. of tubes											
10^{0}	.	.	.	6	8	10	10	10	12	16	16	16
10^{-1}	.	6	6	6	6	6	6	6	6	6	6	6
10^{-2}	.	6	6	6	6	.	.	.	.	.	.	.
10^{-3}	.	6	6	6	6	.	.	.	.	.	.	.
10^{-4}	.	6	6	6	6	.	.	.	.	.	.	.
10^{-5}	6	6	6	6	.	.	.	.	.	.	.	.
10^{-6}	6	6	6	6	.	.	.	.	.	.	.	.
10^{-7}	6	6	6	.	.	.	.	.	.	.	.	.
10^{-8}	6	6	6	.	.	.	.	.	.	.	.	.

Procedure. Roux bottles containing 200 ml. of Tween 80 albumin medium (Medical Research Council, 1948) were inoculated with the strain under investigation and were incubated at 37° for 14 days. The contents of three bottles (600 ml.) was centrifuged for 50–60 min. at 3000 r.p.m., and the deposit resuspended in 30–40 ml. of the medium. Serial tenfold dilutions were then made in Tween 80 albumin medium. From appropriate dilutions volumes of 0·2 ml. were inoculated on to the surface of the tubes of Herrold's medium with dropping pipettes. A typical arrangement of an experiment is shown in Table 1. The tubes were kept in the incubator till the surface of the medium was dry and were then plugged with waxed corks. After 6 weeks' incubation at 37° the number of colonies was counted in dilutions where up to 20 colonies appeared in each tube and a curve was obtained relating surviving organisms to streptomycin concentration.

Isolated colonies were then picked off into tubes of Tween 80 albumin medium. Usually, thirteen colonies were sampled from each streptomycin concentration, but in some of the experiments there was only a smaller number of separate colonies available in the tubes containing the higher concentrations of streptomycin. Strains were subcultured into a second set of tubes of Tween 80 albumin medium and, after 10 days' incubation, sensitivity tests to streptomycin were performed in the same medium by the method recommended by the Medical Research Council (1948) in which an inoculum of 0·02 ml. is used, and the tests are read after 10 days' incubation.

RESULTS

Experiments with the strain Pa were set up on two occasions, and those with Pr and H37Rv on one occasion each. The results of the sensitivity tests on the strains obtained by picking off colonies are given in Table 2*a–d*. With the strain Pa an equal number of colonies was sampled from each streptomycin concentration in Herrold's medium. The numbers of strains inhibited by the same concentration of streptomycin in the liquid medium sensitivity tests were then added up over the whole range of concentrations in the Herrold's medium. These totals, plotted against the inhibitory concentrations in the sensitivity tests, are shown in Fig. 1. Where the number of colonies sampled was not equal (strains Pr and H37Rv) the figures are adjusted so that equal weight is given to the sample from each streptomycin concentration in Herrold's medium. Thus, if ten colonies were sampled from one concentration and thirteen from another the numbers of tests from the latter would be multiplied by 10/13 and the totals added up as before. Since the streptomycin concentrations in

Table 2. *The distribution of streptomycin resistance determined in Tween 80 albumin medium, of cultures from colonies growing in various concentrations of streptomycin in Herrold's medium*

(*a*) Strain Pa, Exp. 1.

Concentration of streptomycin in Herrold's medium (μg./ml.)	Minimal inhibitory concentration of streptomycin in sensitivity tests (μg./ml.) 0·25	0·5	1	2	4	8	16	32	64	128	216	512	1024	2048	>2048	No. colonies sampled
0	1	10	2	.	.	.	.	.	.	.	.	.	.	.	.	13
1	1	10	2	.	.	.	.	.	.	.	.	.	.	.	.	13
2	3	10	.	.	.	.	.	.	.	.	.	.	.	.	.	13
4	2	9	2	.	.	.	.	.	.	.	.	.	.	.	.	13
8	.	.	4	4	5	.	.	.	.	.	.	.	.	.	.	13
16	.	.	3	9	.	1	.	.	.	.	.	.	.	.	.	13
32	.	.	3	10	.	.	.	.	.	.	.	.	.	.	.	13
64	.	.	1	10	.	1	.	.	.	.	.	.	.	.	1	13
128	.	.	.	.	2	1	2	.	4	.	1	.	.	.	3	13
256	.	.	.	.	.	.	.	.	5	5	2	.	.	.	1	13
512	.	.	.	.	.	.	.	.	3	6	1	.	.	.	3	13
1024	.	.	.	.	.	.	.	.	3	3	1	.	.	.	6	13
Totals	7	39	17	33	7	3	2	0	15	14	5	0	0	0	14	

(*b*) Strain Pa, Exp. 2.

Concentration of streptomycin in Herrold's medium (μg./ml.)	Minimal inhibitory concentration of streptomycin in sensitivity tests (μg./ml.) 0·25	0·5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048	No. colonies sampled
0	.	7	6	.	.	.	.	.	.	.	.	.	.	.	.	13
2	2	8	3	.	.	.	.	.	.	.	.	.	.	.	.	13
4	2	9	2	.	.	.	.	.	.	.	.	.	.	.	.	13
8	.	.	3	9	1	.	.	.	.	.	.	.	.	.	.	13
16	.	.	2	10	1	.	.	.	.	.	.	.	.	.	.	13
32	.	.	.	13	.	.	.	.	.	.	.	.	.	.	.	13
64	.	.	2	7	2	.	1	1	.	.	.	.	.	.	.	13
128	.	.	.	.	.	.	.	2	6	.	.	.	.	.	5	13
256	.	.	.	.	.	.	2	3	2	.	1	.	.	.	5	13
512	.	.	.	.	.	.	.	1	2	.	1	.	.	1	8	13
1024	.	.	.	.	.	.	1	1	.	.	.	.	1	.	10	13
Totals	4	24	18	39	4	0	4	8	10	0	2	0	1	1	28	

Table 2 (*cont.*)

(*c*) Strain Pr.

Concentration of streptomycin in Herrold's medium (μg./ml.)	Minimal inhibitory concentration of streptomycin in sensitivity tests (μg./ml.)															No. colonies sampled
	0·25	0·5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048	
0	12	1	.	.	.	.	.	.	.	.	.	.	.	.	.	13
1	12	1	.	.	.	.	.	.	.	.	.	.	.	.	.	13
2	8	4	1	.	.	.	.	.	.	.	.	.	.	.	.	13
4	5	3	5	.	.	.	.	.	.	.	.	.	.	.	.	13
8	.	.	3	9	.	.	.	.	.	.	.	.	.	1	.	13
16	.	.	6	4	2	1	.	.	.	.	.	.	.	.	.	13
32	.	.	2	2	.	.	1	3	1	.	.	.	.	.	4	13
64	.	.	.	.	.	1	.	1	1	.	.	.	.	.	4	7
128	.	.	.	.	.	.	.	1	1	1	.	.	.	.	4	7
256	.	.	.	.	.	.	1	1	.	2	.	.	.	.	1	5
512	.	.	.	.	.	.	.	.	.	1	.	.	.	.	4	5
1024	.	.	.	.	.	.	.	.	.	.	.	.	.	2	2	4
Totals	37	9	17	15	2	2	2	6	3	4	0	0	0	3	19	
Adjusted totals*	11·38	2·91	5·31	4·62	0·62	0·95	1·18	2·86	1·52	2·97	0	0	0	2·38	11·81	

(*d*) Strain H37Rv.

Concentration of streptomycin in Herrold's medium (μg./ml.)	Minimal inhibitory concentration of streptomycin in sensitivity tests (μg./ml.)																No. colonies sampled
	0·12	0·25	0·5	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048	
0	2	11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	13
2	.	1	1	5	5	1	.	.	.	.	.	.	.	.	.	.	13
4	.	.	.	8	2	.	1	.	2	.	.	.	.	.	.	.	13
8	.	.	3	1	1	2	.	.	2	4	.	.	.	.	.	.	13
16	.	.	.	.	.	.	.	4	7	2	.	.	.	.	.	.	13
32	.	.	.	.	.	.	2	1	10	.	.	.	.	.	.	.	13
64	.	.	.	.	.	.	.	.	7	5	1	.	.	.	.	.	13
128	.	.	.	.	.	.	2	1	8	2	.	.	.	.	.	.	13
256	.	.	.	.	.	.	1	4	2	2	.	.	.	.	.	4	13
512	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	8	10
1024	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7	7
Totals	2	12	4	14	8	3	6	10	38	15	1	0	1	1	0	19	
Adjusted totals*	1·08	6·46	2·15	7·54	4·31	1·62	3·24	5·38	15·09	8·08	5·54	0	0·7	0·7	0	14·75	

* For explanation see text.

Herrold's medium are equally spaced, peaks in the curves in Fig. 1 indicate a tendency for strains to segregate into groups with characteristic levels of resistance. It is clear that there are four such peaks. The first corresponds to sensitive strains, the second to slightly resistant strains, the third to moderately resistant strains and the fourth to highly resistant strains capable of growth in 2048 μg./ml.

In Fig. 1 the results of a number of sensitivity tests by the same method on strains derived from patients under treatment with streptomycin are also plotted. One part of the curve is derived from the tests on 192 strains isolated before treatment (Medical Research Council, 1948). The other part is derived from tests on 432 strains obtained from patients during treatment and after a strain at least four times less sensitive than the standard strain H37Rv had been obtained, i.e. after some degree of resistance to streptomycin had appeared in the strains isolated. The results on 398 of these strains were published previously (Mitchison, 1949), but they were then expressed in terms of relative sensitivity to the standard strain. These data again show the presence of four peaks corresponding to those obtained in this paper.

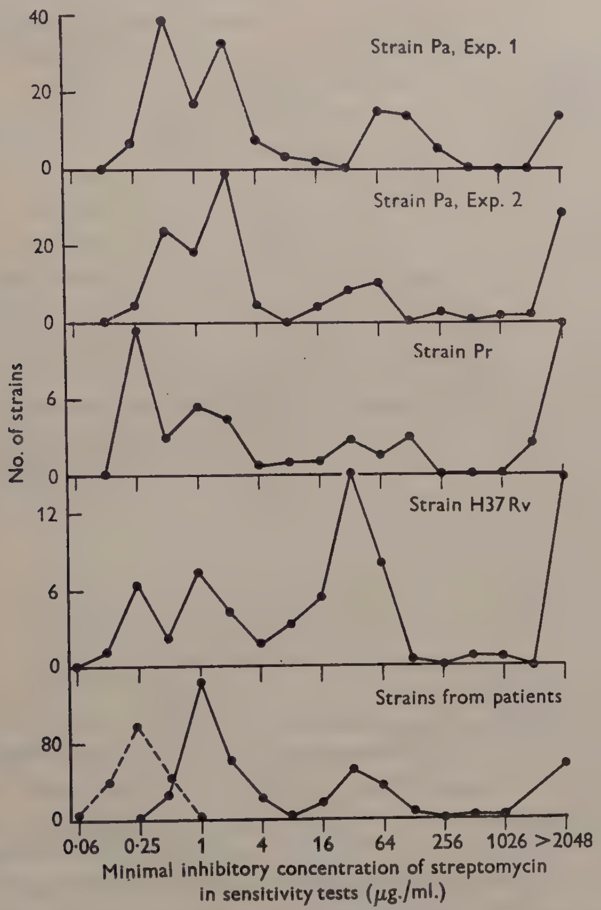

Fig. 1. The distribution of substrains of *M. tuberculosis* derived from Herrold's medium with respect to the minimal inhibitory concentration of streptomycin in the liquid medium sensitivity test. The values for Strains Pr and H37 Rv have been adjusted as described in the text, to allow for uneven sampling of the colonies on Herrold's medium. The strains from patients include 192 (––––) obtained before treatment with streptomycin, and 432 (———) from patients yielding streptomycin-resistant strains.

The curves relating surviving organisms to streptomycin concentrations (survivor curves) are given in Fig. 2. These curves may be interpreted as a series of steps, corresponding to colony counts of sensitive, slightly resistant, moderately resistant and highly resistant strains. As the streptomycin concentration increases, there is first a sharp fall in the number of survivors accompanied by a disappearance of sensitive strains. There is then a relatively flat portion of curve, and slightly resistant strains were obtained from colonies in these tubes. This is followed by another fall in numbers and a second levelling off. Colonies picked from these concentrations usually yielded a mixture of moderately and highly resistant strains. There is considerable variation from experiment to experiment in the concentration of streptomycin in the Herrold's medium which allows growth of colonies yielding strains of any particular level of resistance. In only one experiment, with H37 Rv, were the higher concentrations sufficient to prevent the growth of moderately resistant strains. Medium containing 512 and 1024 μg./ml. yielded highly resistant strains only and it will be seen that again there is another slight fall in surviving organisms between the tubes containing 256 and 512 μg./ml., but that approximately the same numbers survive in 1024 as in 512 μg./ml.

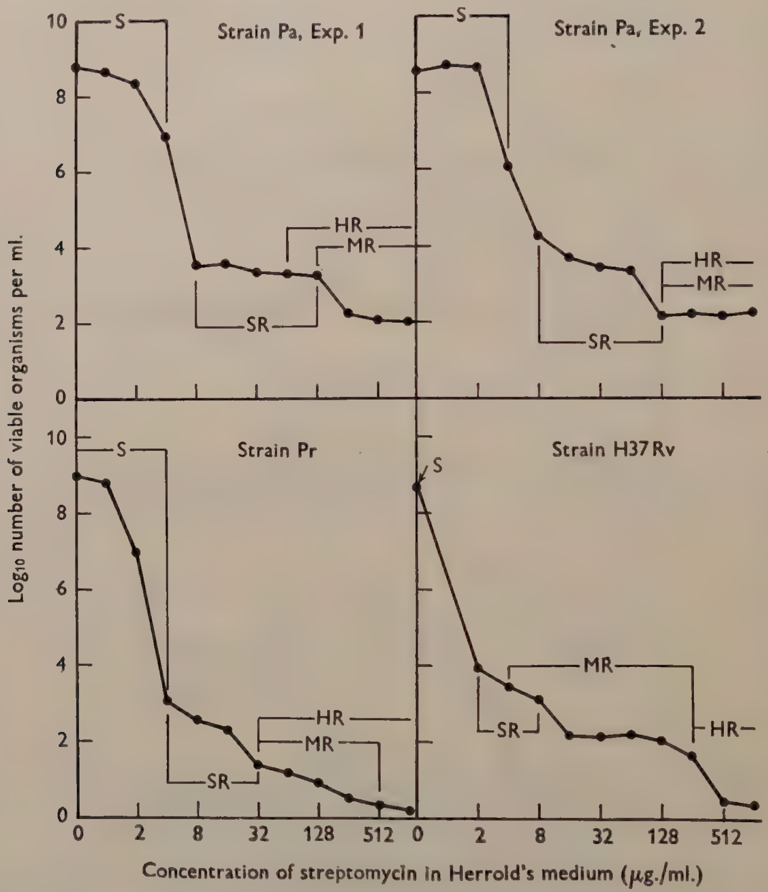

Fig. 2. Survivor curves of *M. tuberculosis* on streptomycin-containing media. Log_{10} number of surviving organisms in different concentration of streptomycin in Herrold's medium. The substrains derived from Herrold's medium were sensitive over the streptomycin concentrations marked S, slightly resistant over the streptomycin concentrations marked SR, moderately resistant over the streptomycin concentrations marked MR and highly resistant over the streptomycin concentrations marked HR.

DISCUSSION

Cultures of tubercle bacilli in Tween 80 albumin medium, although growing in a macroscopically dispersed manner, contain a mixture of separated bacilli and small clumps. The viable counts of these strains, when performed in the absence of streptomycin, will therefore tend to be an underestimate of the number of bacilli present, since some of the colonies will be derived from clumps rather than individual bacilli. However, in the presence of streptomycin only a small fraction of the bacilli are capable of growth and the error introduced by clumping becomes insignificant. For this reason the ratio between sensitive and resistant organisms derived from the survivor curves is likely to be too low, but the ratio between different types of resistant organisms is more likely to be accurate. Bearing this in mind, one can say that, out of a total population of $0{\cdot}4–1{\cdot}0 \times 10^9$ organisms in each ml., somewhere about 100 are slightly resistant, 100 are moderately resistant and 2 are highly resistant. The estimate for the highly resistant variants agrees well with that of Yegian & Vanderlinde. These authors found that colonies growing on medium containing 100 μg. streptomycin/ml. yielded strains capable of growth on 1000 μg./ml., whereas, in the present work, most of the colonies derived from Herrold's medium containing 64 or 128 μg./ml. were moderately resistant and, therefore, inhibited by 1000 μg./ml. This might be due to the presence of Tween 80 in Yegian & Vanderlinde's solid medium which increases the apparent activity of a number of antibiotics including streptomycin (Youmans & Youmans, 1948) and would probably inhibit moderately resistant strains in the presence of 100 μg./ml.

The segregation of streptomycin resistant variants into three groups suggests that there are at least three separate biochemical processes which are interfered with by streptomycin. It is possible that the slightly resistant strains have developed an alternative pathway for one of these processes, but not for the other two, whereas the moderately resistant strains have alternative pathways for two of the processes and the highly resistant strains for all three. It is also possible that the resistance of the slightly resistant and moderately resistant organisms is due to a decreased permeability of the cells as has been suggested for penicillin resistance in yeasts by Maas & Johnson (1949), or to the production of a resistant enzyme as Gots & Sevag (1948) report for sulphonamide resistance.

Oginsky, Smith & Umbreit (1949) consider that streptomycin prevents the formation of an oxaloacetate-pyruvate condensation product. They worked with *Bacterium coli* strains and with tissue homogenates. Highly resistant variants of the same *Bact. coli* strains were unable to affect this condensation. It would be interesting to know whether similar results could be obtained with sensitive and highly resistant tubercle bacilli, and whether the slightly and moderately resistant strains are capable of effecting the condensation.

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United Kingdom Culture Collections

BY S. T. COWAN

National Collection of Type Cultures, Colindale, London, N.W.9

SUMMARY: Old catalogues are useless in trying to locate micro-organisms in culture collections. The addresses are given of the larger culture collections in the U.K., and an indication of the type of micro-organisms each maintains.

Specialized collections exist in different parts of the country, but in the past few have undertaken to distribute cultures except as a personal favour to colleagues working on similar problems. The chief difficulty of those seeking cultures is to know where to write, and what cultures are available. There have been so many changes in the organisms kept in the different collections that pre-war catalogues give no indication of the present whereabouts of the various species. It may be helpful to indicate where different types of micro-organism are now kept.

Algae and Protozoa. Algae, flagellata and ciliosa are kept in Dr E. G. Pringsheim's collection at Cambridge (Collection 1 in Table 1). When application is made for a culture the intended use should be indicated so that the culture may be brought into the appropriate state.

Other laboratories keep Protozoa of interest to medical and veterinary workers, and the addresses can be found in a mimeographed catalogue prepared by the Laboratory Animals Bureau, which can be obtained from the Royal Veterinary College, Royal College Street, London, N.W. 1.

Bacteria. Strains used in microbiological chemistry are kept at Teddington (Collection 2) where they are checked not only for purity but also for their activity as agents for microbiological assays. In the same collection are bacteria used in industrial processes such as the production of dextrans, acetone and vinegar.

Bacteria pathogenic for plants are available from Dr W. J. Dowson's comprehensive collection at Cambridge (Collection 3). Human and animal pathogens and related non-pathogenic species can be obtained from the National Collection of Type Cultures at Colindale (Collection 4). The function of this collection is to supply strains that are culturally and serologically typical; it also acts as a repository for strains that are not fully worked out.

Strains of proven toxigenicity, and in which the different toxins have been identified, are kept at Beckenham (Collection 5) and can be obtained in stable form as desiccates.

Leptospira are technically difficult to keep in pure culture; Dr J. C. Broom has kindly offered to meet requests for these cultures from his collection of thirty-six species. Those who want cultures should write to the Director of the Laboratories (Collection 6).

Fungi. Isolates of taxonomic significance, 'syllabus types' for teaching, plant pathogenic species, and fungi used in industry can be obtained from

Table 1. *Culture Collections in the United Kingdom*

1 Collection of Algae and Protozoa,
Botany School,
Downing Street,
Cambridge.

2 National Collection of Industrial Bacteria,
Chemical Research Laboratory,
Teddington, Middlesex.

3 Collection of Bacteria Pathogenic to Plants,
Subdepartment of Mycology and Plant Pathology,
Botany School, Downing Street,
Cambridge.

4 National Collection of Type Cultures,
Central Public Health Laboratory,
Colindale Avenue,
Colindale, London, N.W. 9.

5 Wellcome Collection of Bacteria,
Wellcome Research Laboratories,
Langley Court,
Beckenham, Kent.

6 Wellcome Laboratories for Tropical Medicine,
Euston Road,
London, N.W. 1.

7 Commonwealth Mycological Institute Collection of Fungus Cultures,
Ferry Lane,
Kew, Surrey.

8 Forest Products Research Laboratory,
Princes Risborough,
Aylesbury, Bucks.

9 Subdepartment of Medical Mycology,
London School of Hygiene and Tropical Medicine,
Keppel Street, Gower Street,
London, W.C. 1.

10 Brewing Industry Research Foundation,
Lyttel Hall,
Nutfield,
near Redhill,
Surrey.

the Commonwealth Mycological Institute (Collection 7). Antibiotic producing strains are also kept, but the collection does not guarantee that they are in the state of maximum productivity.

The wood-rotting fungi form a highly specialized collection sponsored by D.S.I.R. (Collection 8).

Species pathogenic for man and animals were collected by Dr J. T. Duncan and can be obtained from his successor (Collection 9).

Viruses and bacteriophages. None of the collections maintains viruses or bacteriophages, but the N.C.T.C. can obtain these for experienced workers from specialized laboratories.

Yeasts. Pathogenic yeasts can be obtained from Collection 9, non-pathogenic species from the new Brewing Industry Research Foundation (Collection 10). This collection is not limited to species used by brewers.

Lists of cultures

Many of the collections mentioned are preparing catalogues giving details of the strains they maintain, but until these have been printed the gap will be filled by a *List of Species* appended to a Directory of Culture Collections in the United Kingdom and Crown Colonies. This will be published by His Majesty's Stationery Office on behalf of the British Commonwealth Collections of Micro-organisms, an organization set up on the recommendation of a specialist conference (Report, 1947). National committees in each of the Dominions and the United Kingdom have collected information about collections large and small, and within the next few years a series of directories will provide information on microbiological cultures available in the Commonwealth.

REFERENCE

Report (1947). *Specialist Conference on Culture Collections of Micro-organisms.* (S.O. Code No. 47–139.) London: H.M.S.O.

(*Received* 13 *March* 1951)

No. 3, *Volume* 5 *of the Journal of General Microbiology was issued on* 21 *August*, 1951

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The Influence of Acridines on the Interaction of *Staphylococcus aureus* and Staphylococcus *K* phage

By J. E. HOTCHIN

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SUMMARY: The inhibition of multiplication of staphylococcus *K* bacteriophage by six acridines and two amidines has been studied using mass lysis and one-step growth curve techniques. The effect of the different inhibitors upon the growth rate of the organisms was found to vary from below 5 to 100 % inhibition at a concentration sufficient to double lysis-time. Results of one-step growth curves showed a progressive decrease in burst size with increasing concentrations of inhibitor, and gave no evidence for any variation in sensitivity to inhibitor during the latent period. Using higher concentrations of inhibitor a zone was found where accelerated lysis occurred with loss of phage.

The bacteriophages are being studied increasingly in experimental systems for the investigation of the factors determining the interaction between virus and host cell. The aim of the work described here was to ascertain the extent to which the lysis of a pure culture of *Staphylococcus aureus* by a given staphylococcal phage is affected by acridines, certain of which have been reported (Fitzgerald & Babbitt, 1946) to inhibit lysis of an *Escherichia coli* phage system. When an inhibition of mass lysis had been demonstrated in the *Staph. aureus* phage system, more detailed studies were made using one-step growth curves. An attempt was made to throw some light upon the processes of bacteriophage multiplication from observations of the way in which the inhibitors acted.

MATERIALS AND METHODS

Organisms. All experiments were performed on the Oxford 'H' strain of *Staph. aureus* (N.C.T.C. No. 6571A), grown on slopes of 2 % agar in tryptic digest broth (Hartley, 1922), and subcultured daily.

Bacteriophage. The anti-staphylococcus phage staphylococcus *K* originally obtained from Dr A. P. Krueger was used throughout.

Inhibitors. Five acridine compounds, two compounds with similar structure (rhodamine and fluorescein) and two with similar action but different structure (propamidine and pentamidine), were tested (see Table 1). Concentrations are expressed throughout in g.mol./l. (M) and micromols/l. (μM), for convenient comparison. Solutions (0·01 M) were made up in distilled water and sterilized by filtration through 0·7 μ. Gradocol membranes.

Bacterial growth. Two methods of estimation were used: the plate count of colonies of viable organisms, and measurement of the turbidities of cultures using a Hilger 'Biochem' absorptiometer.

Bacteriophage growth. Phage suspensions were titrated by plaque-counts on 1 % agar plates.

One-step growth curves. The technique used was that devised by Delbrück & Luria (1942) with modifications to permit the application and removal by dilution of inhibitor for varying periods during the latent period, and to permit several experiments to be run concurrently. The latter was achieved by staggering the sampling times by half-minute intervals. A final 10^{-6} dilution of the experimental tube permitted samples to be plated directly and gave a convenient count up to 200 plaques. The action of the inhibitor was arrested by a 10^{-2} dilution. Initial concentrations of viable organisms and phage in the experimental system were: organisms 3×10^7/ml.; bacteriophage 4×10^7/ml. Adsorption for 5 min. gave in control tubes an infective ratio of less than 1 and a burst size of about 60. Samples of the same batch of broth were used throughout to ensure uniformity of the nutrient medium; experience showed this to be most important.

Bacterial lysis. Degrees of mass lysis were measured photoelectrically. Turbidimetric readings were usually taken over a period of *c.* 6 hr. and then plotted.

Influence of inhibitor on lysis. Organisms in the logarithmic phase of growth in broth were spun down and resuspended in fresh broth to provide a suspension of standard turbidity. Five concentrations of inhibitor were used in duplicate for each experiment, with two control tubes, one for organism growth and one for lysis by phage. To half the tubes sufficient phage was added in 0·1 ml. broth to give complete lysis in the control tube in 2 to 4 hr. The other six tubes received no phage, to provide curves for the direct effect of inhibitor upon bacterial growth. Immediately after addition of phage, all tubes received 2 ml. of the standard suspension of organisms, and were incubated at 37°. The initial viable count of bacteria in these experiments was $1{\cdot}5 \times 10^8$ organisms/ml., and the initial phage titre was 1×10^8 particles/ml.

RESULTS

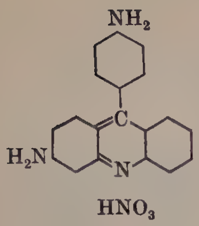
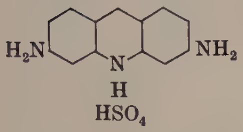
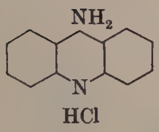
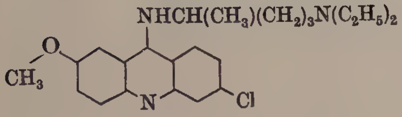
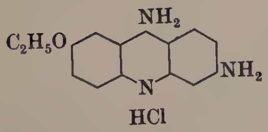
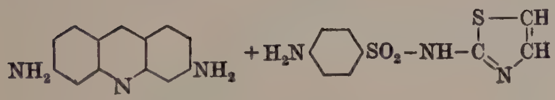
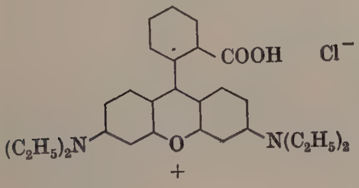
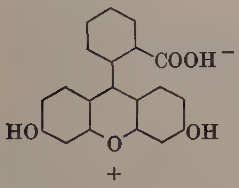
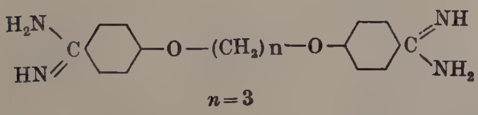
The effect of acridines on staphylococci

All inhibitors were first tested for their power to prevent completely the growth of a small inoculum of *Staph. aureus* in broth during 24-hr. incubation. The minimal bacteriostatic concentration was taken as a guide for the concentrations to be used later with the phage+host-cell systems. Graded effects upon the rate of growth of the organisms were obtained in all cases with concentrations of inhibitor below the level needed to prevent growth. The results of these experiments are summarized with those of lysis experiments, in Table 2, cols. 3 and 4.

The action of acridines on staphylococcus K bacteriophage

The effects of most of the inhibitors were tested upon staphylococcus *K* phage alone, at 37° in broth. Previous results obtained by Fitzgerald & Babbitt (1946) and by Foster (1948) showed the direct inactivation of coli *T* phages by acridines to be negligible at concentrations which inhibited the growth of phage in the presence of its host organism. This was confirmed

Table 1. *Structural formulae of compounds tested*

	Phosphine IIc (C.I.>93) (ICI)
	Proflavine (Boots)
	5-Aminoacridine (Boots)
	Mepacrine (HK)
	Rivanol (HK)
	Flavazole (Boots)
	Rhodamine B (BDH)
	Fluorescein (Harr.)
	Propamidine isothionate (M & B)
As above $n=5$	Pentamidine isothionate (M & B)

Sources of compounds used: (ICI)=Imperial Chemical Industries Ltd.; (Boots)=Boots Pure Drug Co.; (HK)=Dr H. King; (BDH)=British Drug Houses Ltd.; (Harr.)=Harrington Bros.; (M & B)=May & Baker.

Table 2. *Critical concentrations and pH values of inhibitors affecting* Staph. aureus *and staphylococcus K phage in broth medium pH 7–7·4*

1	2	3	4	5	6	7
Inhibitor	pH value at 0·01M in water	Concentration tolerated by logarithmically growing organisms (μM)	Completely inhibitory concentration for growth of organisms (μM)	Minimum concentration [X] at which lysis time is doubled (μM)	Degree of inhibition of organisms growth caused by [X] (%)	Minimum concentration directly inactivating phage (in 3 hr. at 37°) (μM)
Phosphine IIc	2·72	6	50	>50	100	>2500
Proflavine	7·20	12	100	12	<5	>2500
5-Aminoacridine	5·93	24	160	50	5	312
Mepacrine	4·08	250	Not determined	375	25	1250
Rivanol	5·36	3	37	10	25	20
Flavazole	7·64	12	Not determined	>12	5	Not determined
Rhodamine B	2·55	3	400	50	40	>2500
Fluorescein	7·58	170	≫2500	>2500	Not determined	Not determined
Propamidine (isothionate)	6·25	6	50	12	10	2500
Pentamidine (isothionate)	6·68	15	50	>50	100	Not determined
Parent acridine	3·45	100	2500	625	65	>1250

Values in column 6 were derived from individual turbidity-time curves.

using T_2 phage, which was not affected by 1000 μM. acridine at 37° in broth for 20 hr. Staphylococcus K phage, however, was more susceptible to inactivation by acridines than was T_2. The relative inactivating concentrations of most of the inhibitors studied are shown in Table 2, col. 7.

The effect of acridines on the lysis of staphylococci by phage

Turbidimetric results. A typical series of turbidity-time curves obtained in experiments with 5-aminoacridine (Figs. 1 and 2) illustrates the effect of various concentrations of this compound on the growth of staphylococci and their lysis by phage. Similar curves were determined for each of the substances tested. The results are summarized in Table 2, cols. 5 and 6. It is interesting to compare the figures for maximum concentration of inhibitor tolerated by the organism without noticeably (within 5 %) altering its logarithmic growth rate (column 3), and the minimum concentration [X] of inhibitor required to double the lysis time (column 5). It is evident that inhibition of lysis and of the growth of the organism are not always parallel. A strong inhibitory effect on lysis is almost always associated with some interference with the metabolic processes of the host as shown by a measurable decrease in growth rate, the degree of interference varying with the compound. Proflavine, for example, inhibited lysis significantly at a concentration where only a very slight inhibition of host multiplication was detectable.

The effects of increasing the concentration of inhibitor substance on the shape of the curves for growth and lysis were studied, and some interesting

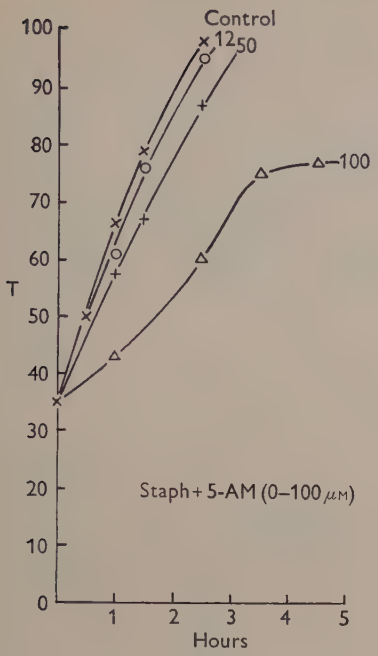


Fig. 1

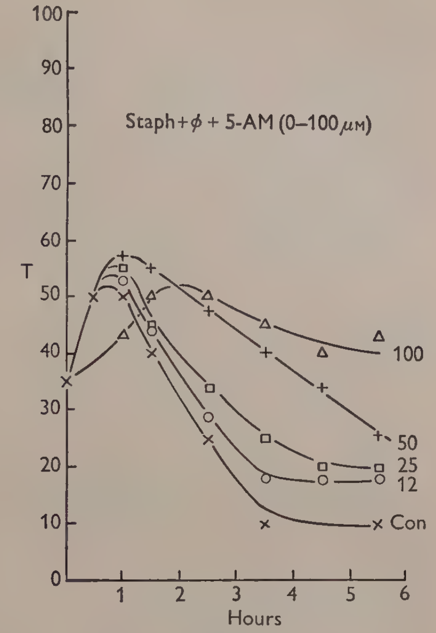


Fig. 2

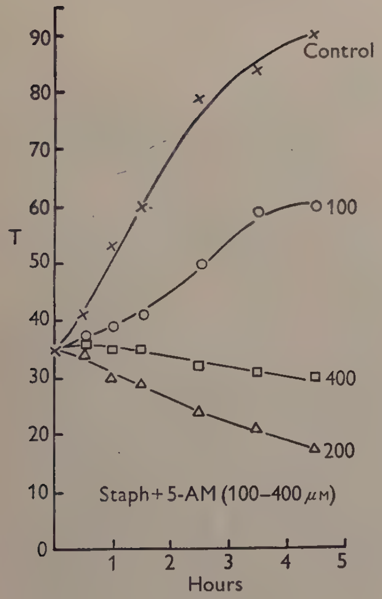


Fig. 3

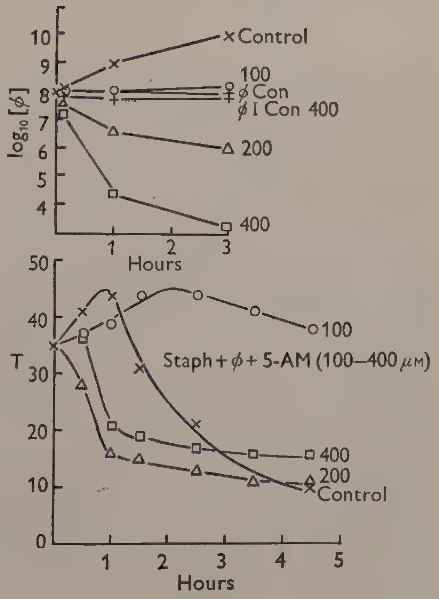


Fig. 4

Figs. 1–4. Effects of 5-aminoacridine upon turbidity-time curves of *Staph. aureus* 'Oxford' cultures with and without phage. Figures show concentrations of acridine in μM. For abbreviations see p. 618.

new phenomena observed. Figs. 3 and 4 show the curves obtained in such an experiment, using 5-aminoacridine. The concentration of 100 μM. caused long inhibition of lysis, but the curve obtained with a twofold increase of inhibitor to 200 μM. was significantly different. Here, lysis was greatly accelerated, beginning at once and in 1 hr. reaching a degree which was only reached in 3 hr. by the control. The 400 μM. tube showed a similar curve but the effect was less well-marked, with a shift back towards the control. The accompanying changes in titres of available phage are shown in the upper part of Fig. 4.

The growth of the organisms alone was affected in a parallel manner (Fig. 3): 100 μM. inhibitor gave approximately 50 % inhibition of growth. With 200 μM. however there was no growth, but the turbidity of the culture steadily diminished. Increasing the concentration of 5-aminoacridine still further, to 400 μM., produced a similar but smaller effect.

Appreciable multiplication of phage occurred in the control lysis tube, and, to a lesser extent, in the presence of 100 μM. inhibitor. At concentrations 200 and 400 μM., the titre of phage fell off steeply. In the absence of staphylococci these concentrations of inhibitor produced no comparable change in the titre of phage over the same period of incubation. The direct effect of 312 μM. (see Table 2, col. 7) only accounts for a small fraction of the inactivation. Apparently, therefore, phage at low concentration can lyse organisms which have their growth processes entirely inhibited, but the phage itself does not multiply under these conditions. This accelerated lysis and decrease in turbidity of organisms was also observed with propamidine.

Results using one-step growth curves

The curves in Fig. 5 show the effects of different concentrations of 5-aminoacridine upon the one-step growth curve of staphylococcus *K* phage. The system was exposed to the inhibitor from the fifth minute onwards. A progressive increase in concentration of inhibitor caused a progressive decrease in burst size, until at 100 μM. no increase in phage was detectable. This level corresponds to the 'completely inhibitory' concentration of Foster (1948). In an analysis of phage infection, its initial adsorption by the organism may be studied, but from there onwards until new phage is liberated during lysis, what happens within the cell is largely unknown. It was thought that an investigation of the effect of allowing the inhibitor to act for a given time at different stages within the latent period might prove illuminating and perhaps indicate differences in host-sensibility at different phases of phage multiplication. The result of the first experiment of this kind is shown in Fig. 6. A concentration of 50 μM. 5-aminoacridine was added at progressively later stages in the latent period. The burst size decreased in value proportionately to the time of contact with the drug. The curves indicate that the latent period was unaffected, and this was confirmed in other experiments.

The effect of time of exposure of the multiplying phage to 50 μM. inhibitor was investigated further by arresting the action of the drug after varying periods within the latent period. This was achieved by finally diluting the inhibitor one hundredfold. The inhibitor decreased the burst size and extended the latent period proportionately to the duration of its action. The effect on burst size was less than when the inhibitor was added and thereafter allowed to operate continuously during the latent period. It must be remembered in the latter case that besides acting in the latent period, the inhibitor continued to act during the rise period, which in the inhibited system was sometimes twice as long as the usual 10 min. The shorter exposure of the system, when inhibition was removed, allowed its recovery from the effects of the inhibition. The amount of recovery possible appeared to be directly proportional to the period remaining before lysis was due to be complete, i.e. the end of the rise period.

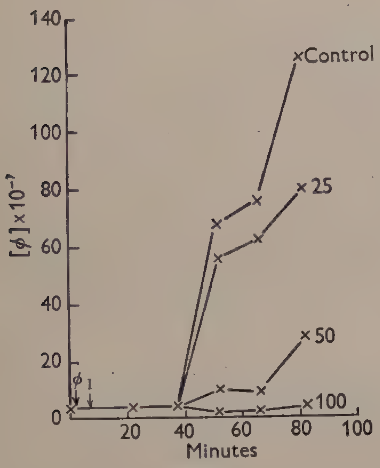


Fig. 5

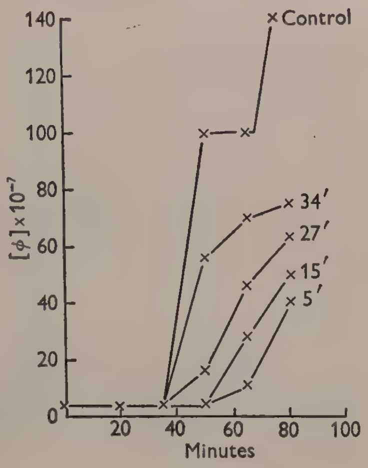


Fig. 6

Effects of 5-aminoacridine upon one-step growth curves of staph. *K* phage.

Fig. 5. Figures show concentration of 5-aminoacridine in μM.
ϕ = Time of infection of *Staph. aureus* 'Oxford' with ϕ.
I = Time of addition of inhibitor.

Fig. 6. Figures show times of addition of 5-aminoacridine to give 50 μM. concentration. Infection with ϕ was at zero time.

When the system was exposed to inhibitor for 10-min. periods, during each of three consecutive thirds of the latent period, no differences in the amount of inhibition produced could be detected.

A similar comparison was made of the effects of the sub-inhibitory concentration 50 μM. with those of the completely inhibitory concentration of 200 μM. In each case the inhibitor was allowed to act for a 10-min. period in the middle of the latent period (Fig. 7). The capacity of the system to recover is well illustrated. In tube *B* where the concentration was 50 μM. recovery is almost complete. At the concentration of 200 μM., however, the burst size is decreased and the latent period extended by an interval equal to that of the exposure period. Reference to Fig. 1 shows that at this concentration the multiplication of organisms is completely inhibited. Also at this concentration accelerated lysis is at a maximum (see Fig. 4) and is invariably accompanied by a decrease in phage titre.

The effect of nucleic acids on the inhibition by acridines of the lytic action of phage and the growth of organisms

The observation of Fitzgerald & Lee (1946), of Smith (1949) and of Foster (1948) that yeast ribonucleic acid counteracts the effects of acridines upon lysis by phage was confirmed for *Staph. aureus* and staphylococcus *K* phage. Commercial preparations of nucleic acid were added before the acridine. Ribonucleic acid (RNA) was inferior to thymus deoxyribonucleic acid (DNA) in counteracting the effect of phosphine IIc and 5-aminoacridine. When the w/v concentration of DNA was 25 times that of the acridine, the inhibition of lysis by 5-aminoacridine was completely antagonized, whereas RNA did not antagonize unless its concentration was 100 times that of the acridine. Reversal of the inhibition of growth of organisms by acridines was obtained by adding the nucleic acid after an interval but recovery only occurred gradually after a long lag and for this reason was not demonstrable with a phage-inhibitor-organism system.

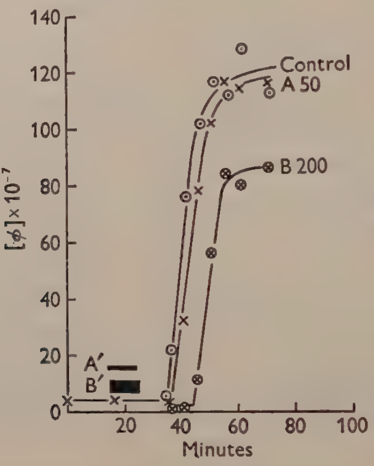


Fig. 7. The effect of 50 μM. 5-aminoacridine (*A*) and 200 μM. 5-aminoacridine (*B*). Exposure time to inhibitor during the latent period is represented as *A′* and *B′*.

DISCUSSION

It is essential, when studying whether or not selected compounds inhibit or modify the interaction of viruses with their host cells, to distinguish between the direct effects of the compound on the host and on the outcome of the infection, so that the therapeutic value of that compound against virus infection may be assessed. The compounds studied here were without effect on the free phage and on its adsorption by the cell at concentrations completely inhibitory for the organisms; the inhibition observed therefore occurred at some point in the reactions involving virus synthesis or release.

It was found that with a compound active against the phage a concentration of this compound below that which is detrimental to the growth of the host organism causes a decrease in the average phage multiplication/cell, as revealed by a fall in burst size. As the concentration is raised further the burst size progressively diminishes with little, if any, change in the latent period of infection. With the inhibitor at a concentration where definite inhibition of the growth of the host organism occurs, the latent period of infection is prolonged and phage multiplication ceases, as indicated by zero burst size. The one-step growth curve then suggests an indefinitely prolonged latent period. When, however, such an inhibited system is diluted into broth containing no inhibitor, then lysis will occur with a high burst size. It appears that when the growth processes of the host cell are arrested by inhibitor, that compound thereafter renders the cell more susceptible to lysis either through the agency of phage itself or by the combined effects of phage and autolytic enzymes, so that rapid lysis occurs with loss of infecting phage.

These observations on the inhibition of the interaction of phage and host staphylococci by acridines are in keeping with those of Fitzgerald & Babbitt (1946) and of Foster (1948), carried out with *Esch. coli* and the *T* series of phages. Their experiments were made in chemically defined medium; the present ones were all performed in broth, which gives a well-defined latent period and a much sharper rise period, so that a more clear-cut picture of the inhibitory effect in one-step growth curves was obtained. Smith (1949) reported results with phosphine GRN and acriflavine on several organisms, including two strains of *Staph. aureus*, and a polyvalent staphylococcus phage preparation. The concentrations he found to be inhibitory to phage multiplication are similar to those used here for the same or closely allied acridines. I did not observe the stimulation of lysis by phosphine at low concentrations reported by Smith with phosphine GRN, but phosphine IIc caused accelerated lysis at higher concentrations.

The fact that no variation in sensitivity was found during the latent period suggests either that phage multiplication proceeds by single fission continuously susceptible to acridine inhibition, or that the normal continuous production of some essential intermediate or precursor substance by the host organism is progressively retarded by the inhibitor with a consequent decrease in phage multiplication, as evidenced by the one-step growth curves. The latter process is considered to be the more likely explanation of the mode of action of the acridines, from the results of these experiments. But it must be remembered that the technique used measures only mature 'viable' phage particles; any phage particles formed during inhibition that were altered so as to render them non-viable would give no indication of their presence.

It is believed that the phenomenon here called accelerated lysis has not previously been described in connexion with high concentrations of acridines or propamidine. It resembles the phenomenon described by Cohen (1949) in which *Esch. coli* was rapidly lysed without phage increase when its respiration was prevented by nitrogen or cyanide. Elford (1948) recorded the accelerated lysis of *Staph. aureus* in the presence of penicillin; this occurred with the production of active phage which had been able to multiply, thus differing from the accelerated lysis due to acridines whereby phage is lost. The numbers of phage particles in the above experiments were far too low for the effect to be due to 'lysis from without' as described by Delbrück (1940).

The close similarity of the action of the acridines and propamidine as phage inhibitors is interesting as it is in accordance with the work of Gotts & Sevag (1948) who showed very close parallelism between the effects of these two drugs on the dehydrogenase inhibition of pneumococci.

It is a pleasure to thank Dr W. J. Elford for his invaluable advice and criticism in the course of this work.

Abbreviations used in figures

ϕ = Bacteriophage.
$[\phi]$ = Bacteriophage concentration, per ml.
5-AM = 5-aminoacridine.
T = Units of turbidity.
μM = Micromolar concentration.
Staph = *Staphylococcus aureus* 'Oxford.'
ϕ *Con* = Phage control.
ϕ *I Con* = Phage plus Inhibitor control.

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Alternaric Acid; a Biologically Active Metabolic Product of *Alternaria solani* (Ell. & Mart.) Jones & Grout; its Production, Isolation and Antifungal Properties

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SUMMARY: When *Alternaria solani* is grown on any one of a variety of defined culture media, an antibiotic metabolic product, alternaric acid, accumulates in the medium. The quantity of alternaric acid produced is directly related to the amount of mycelium formed; good yields of alternaric acid are obtained on any medium which supports good growth. Optimal media contain high concentrations (7·5% (w/v) or more) of sucrose, which is better than any other carbon source tested. Nitrogen may be supplied as nitrate or casein hydrolysate; ammonia as nitrogen source is equally good when supplied in conjunction with a suitable organic acid, 0·25% (w/v) acetic acid being particularly favourable.

The antibiotic is isolated from optimal media by extraction with chloroform after adjustment to pH 3·5; the solvent is then evaporated and the residue recrystallized from benzene. Yields of the order of 150 mg./l. are obtained.

Alternaric acid is not antibacterial. Germination of spores of some fungi (e.g. *Absidia glauca, Myrothecium verrucaria*) is prevented by 1 μg./ml. or less of alternaric acid. Germination of spores of other fungi (e.g. *Botrytis allii*) is unaffected by concentrations as high as 100 μg./ml., but extension of the germ-tubes is markedly retarded shortly after germination. This secondary retarding effect may be produced by very low concentrations; 0·01 μg./ml. produced an obvious effect with *B. allii.*

In a preliminary publication (Brian, Curtis, Hemming, Unwin & Wright, 1949) a metabolic product of *Alternaria solani* was described which was both antifungal and phytotoxic. The latter property was of particular interest in so far as it might be concerned in the etiology of plant diseases caused by *A. solani.* The present account deals with the production and isolation of alternaric acid and with its antifungal properties. Its phytotoxic properties and its possible role in diseases caused by *A. solani* will be reported later.

MATERIAL AND METHODS

Antagonism in agar culture. Detection of antagonism in agar has been carried out by an improvement of the 'streak-test' previously described (Brian & Hemming, 1947). Four test organisms are used, three bacteria (*Bacterium coli, Salmonella typhi* and *Staphylococcus aureus*) and one yeast-like fungus (*Candida albicans*). In the improved method these organisms were grown on a semi-solid medium containing 2% (w/v) sodium alginate; the cultures of each organism were thoroughly mixed and streaked on the agar plates, on which colonies of one or other of the *Alternaria* strains were already established, by expressing from a sterile hypodermic syringe. This method of streaking is far more rapid than streaking infected broth by means of a loop, and gives more regular streaks. Antagonism in agar to the fungus *Botrytis allii* has been tested by the 'reversed agar' method of Jefferys (1948).

Assays of antifungal activity. A spore-germination test with conidia of *B. allii* was used for routine assays. The test was set up as previously described (Brian & Hemming, 1945) but was necessarily read differently. It is shown in this paper that the antifungal substance produced by *A. solani* does not inhibit germination of *B. allii* spores but causes a characteristic stunting of the germ-tubes. Accordingly, the procedure has been to assay a series of twofold dilutions to determine the greatest dilution in which at least 90 % of the germ-tubes do not exceed 200 μ. in length; normal germ-tubes under the same conditions are at least 400 μ. in length. In the first few dilutions of a series, though percentage germination is not affected or, at most, only slightly decreased, the mean germ-tube length may be no more than 20 μ. The antifungal activity determined in this way is presented in this paper as the number of twofold dilutions made before the limiting effect, described above, is reached; in effect, therefore, this 'activity index' is proportional to $\log_2$ of the concentration of active material.

Methods of culture. In experiments which did not involve extraction of the alternaric acid produced, cultures were grown on 250 ml. lots of medium in 'Glaxo' culture vessels (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944). Samples of the underlying medium were aseptically withdrawn periodically for assay. In general, six or more cultures were set up on each medium in an experiment, samples from each vessel in each set of six being bulked for assay. At the conclusion of each experiment the mycelium from each set of six vessels was filtered off, dried at 100° and weighed. Where larger quantities of material were needed, for extraction of alternaric acid, earthenware culture vessels, each containing 0·5–1·0 l., were used. All cultures were grown at 25°. None of the strains of *A. solani* used in this study produced conidia without special treatment; consequently, mycelial inocula have been used.

Origin of cultures. Eleven cultures were studied, but results of five only are reported here in any detail; their provenance is described below:

No. 159. *Alternaria* sp. (NCTC 1007); originally isolated from rotting canvas by Lorrain Smith, 1921.

No. 407. *A. solani* (Ell. & Mart.) Jones & Grout; strain K 30; obtained from R. V. Tipler, Hawthorndale Laboratories, Jealott's Hill Research Station. This was a subculture from a strain (IBPG 7) isolated from tomato fruit, 1946, at the Imperial Bureau of Plant-breeding and Genetics, Cambridge.

No. 408. *A. solani* (Ell. & Mart.) Jones & Grout; strain K 31; obtained from R. V. Tipler, Hawthorndale Laboratories, Jealott's Hill Research Station. This was a subculture from a strain (IBPG 14) isolated from tomato fruit, 1946, at the Imperial Bureau of Plant-breeding and Genetics, Cambridge.

No. 483. *A. porri* (Ell.) Sacc.; Neergaard's strain from Centraalbureau voor Schimmelcultures, Baarn, Holland.

No. 484. *A. porri* (Ell.) Sacc. f. sp. *solani* (E. & M. pr. sp. Neergaard); Neergaard's strain from Centraalbureau voor Schimmelcultures, Baarn, Holland.

Of these, No. 159 is a saprophytic species quite distinct morphologically from the remainder. The other cultures of *A. solani* and *A. porri* are very similar; Neergaard (1945) has proposed to group *A. porri*, *A. solani* and *A. dauci* in one species as *formae speciales* of *A. porri*. Of these strains, Nos. 407 and 483 are strongly chromogenic, producing abundant wine-red pigment on Czapek medium.

RESULTS

Antagonism in agar culture

In agar streak tests *A. solani* No. 407 showed pronounced antagonism to *Staph. aureus* and *Bact. coli*; no other strains showed activity of any kind. All showed activity against *B. allii* in the reversed agar test. Complete lack of activity in agar streak tests has been previously reported for another strain of *A. solani* (Brian & Hemming, 1947).

On the basis of these results, strain 407 was at first selected for further study, the intention being to isolate the antibacterial substance. Cultures were grown on a number of liquid media and assayed for antibacterial activity (serial dilution assays with *Staph. aureus* and *S. typhi*, cylinder plates assays with *B. subtilis*) and antifungal activity. Under these conditions no antibacterial activity was detected but, even at great dilutions, the culture filtrates produced a characteristic 'stunting' of *B. allii* germ-tubes. Attention was diverted to isolation of the substance responsible for this effect and a study of the cultural conditions under which it is produced, using the method of assay described in an earlier paragraph.

Comparison of strains in liquid culture

It now seemed desirable to re-examine all the cultures for production of this factor which caused stunted germ-tubes. Table 1 shows the results of such a comparison. Cultures were grown on medium N (10 % glucose + potassium nitrate + mineral salts) and medium T (10 % glucose + casein hydrolysate + mineral salts); detailed compositions of these media have been given previously (Brian, Curtis & Hemming, 1947). All the strains grew more rapidly on medium T and the activity of culture filtrates was somewhat enhanced in the case of strains 407, 408 and 483. Negligible activity was recorded on both media for strains 484 and 159.

On the basis of this and similar experiments, all of which confirmed the high productivity of strain 408, that strain was chosen for intensive study.

Table 1. *Antifungal activity in cultures of four strains of* Alternaria *on two media (N and T)*

		Days growth					
		4	7	12	17	21	25
		Activity index					
Medium	Strain						
N	408	—	1	7	10	12	12
	407	—	—	5	5	5	6
	483	1	1	1	2	3	4
	484	1	2	1	1	2	2
	159	—	2	2	2	1	—
T	408	—	8	11	14	15	14
	407	—	1	6	8	7	8
	483	1	2	5	5	5	6
	484	—	2	1	2	2	2
	159	1	1	—	—	1	—

Nevertheless, many experiments described below were also confirmed using strain 407, and, as will be seen, the same active material, alternaric acid, was obtained from both strains.

Relation between medium composition and antifungal activity

In preliminary experiments it was found that the antifungal substance was freely formed in a simple glucose+nitrate+mineral salts medium (N). The first studies on medium composition described below are concerned with derivatives of this simple medium in which the nature and concentration of the carbon source and the initial pH of the medium were varied. Subsequently, variation of the nitrogen source and the addition of organic acids were investigated. Strain 408 was used throughout; occasionally strain 407 was included for comparison but results with that strain are not described in detail here.

Glucose concentration. The results of a typical experiment are given in Table 2. It will be seen that both the weight of mycelium produced and the maximum activity titre were directly related to the glucose concentration in the medium. The relation between glucose concentration and pH drift was less simple. In media with 1·0 % or 2·5 % glucose the pH rose rapidly in the first twelve days from 4·8 to about 8·4 and thereafter showed relatively little change. In media with higher glucose concentration the rise was less rapid after the eighth day so that on the twelfth day the pH was around 7·8; thereafter the pH remained steady or fell slightly in the medium with 10 % and 15 % glucose but fell rapidly in the 5 % glucose medium before reaching a steady level. This anomalous behaviour of media with intermediate glucose concentrations has been observed on several occasions and is seen even more strikingly in Table 3, which describes a similar experiment in Czapek medium, which contains potassium chloride in addition to the salts present in medium N.

Table 2. *Effect of glucose concentration in medium N on antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani

	Days growth						
	4	8	12	15	19	23	Final dry wt. of mycelium (g.)
Glucose (%, w/v)	Activity index						
1·0	—	4	5	6	6	7	1·4
2·5	—	5	8	10	11	10	5·9
5·0	—	6	8	11	11	12	16·2
10·0	—	6	9	12	12	13	21·2
15·0	—	7	10	12	13	14	31·1

	Initial pH	pH					
1·0	4·8	5·8	7·6	8·5	8·4	8·7	8·8
2·5	4·8	5·9	7·8	8·3	8·0	8·2	8·6
5·0	4·8	5·7	7·5	7·6	6·7	6·3	6·6
10·0	4·8	5·7	7·1	7·8	7·3	7·2	7·6
15·0	4·8	5·4	7·4	7·8	7·4	7·3	7·4

In this experiment (Table 3) the relationship between glucose concentration, antifungal activity and weight of mycelium is the same as in the experiment with medium N (Table 2) but the pH drift is surprisingly different. In this case, whereas the media with low glucose (1 % and 2·5 %) show a pH rise after a slight initial fall, those with high sugar fall considerably before reaching a steady level, but again the fall is greatest in the medium with 5 % glucose. Both these experiments were repeated with strain 407. Similar results were obtained, though the maximum levels of antifungal activity were much lower.

Table 3. *Effect of glucose concentration in Czapek medium on antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani

	Days growth							
	8	11	15	18	22	26	30	Final dry wt. of mycelium (g.)
Glucose (%, w/v)	Activity index							
1·0	—	1	3	4	7	8	6	3·5
2·5	—	1	6	8	9	10	7	7·8
5·0	—	3	8	9	11	11	11	17·4
10·0	3	5	8	10	12	11	11	23·3
15·0	1	5	10	11	12	12	12	28·1

	Initial pH	pH						
1·0	5·6	5·9	5·3	7·2	7·4	8·0	8·3	8·1
2·5	5·5	6·0	4·7	4·4	4·6	5·1	6·1	7·0
5·0	5·4	6·0	4·5	3·6	3·3	3·5	3·4	3·7
10·0	5·0	6·1	4·9	4·4	4·0	3·9	3·8	4·2
15·0	4·9	5·9	6·4	4·9	5·0	4·6	4·4	4·3

Table 4. *Effect of initial pH of medium N* (10 %, *w/v glucose*) *on antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani

	Days growth							
Initial pH	6	9	13	16	20	26	30	Final dry wt. of mycelium (g.)
	Activity index							
4·0	3	8	9	12	14	15	14	29·7
5·0	4	9	11	14	14	14	15	29·5
5·5	3	7	10	14	15	15	15	30·8
6·6	3	7	13	15	15	15	15	29·8
7·6	—	—	7	11	14	15	15	24·8
	pH							
4·0	5·9	6·6	7·2	7·4	7·8	7·6	8·0	
5·0	6·2	6·6	7·1	7·5	7·4	7·3	7·7	
5·5	5·8	6·4	7·3	7·6	7·9	7·7	7·9	
6·6	6·7	7·1	7·7	7·7	8·1	7·8	8·2	
7·6	7·3	7·2	6·8	7·0	7·3	7·2	7·4	

Initial pH of the medium. The results of an experiment where medium N (10 % glucose) was initially adjusted, with KOH or HCl, to a range of pH values between 4·0 and 7·6 are recorded in Table 4. The pH of all media followed a similar course after the thirteenth day of fermentation; the final titres of antifungal activity and the dry weight of mycelium formed were similar in all media except that initially adjusted to pH 7·6. In that medium growth was initially slow, development of antifungal activity was correspondingly retarded and the final dry weight of mycelium formed was somewhat less than on the other media. In all subsequent experiments the medium has been initially adjusted to pH 5·5–6·5.

Table 5. *Effect of carbon source* (10 %, *w/v*) *in medium N on antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani

		Days growth						
Carbon source		7	11	15	18	21	25	Final dry wt. of mycelium (g.)
		Activity index						
Glycerol		—	1	3	5	8	8	12·4
Galactose		—	2	3	4	8	10	13·6
Glucose		3	6	9	8	10	13	17·9
Lactose		—	2	5	9	12	14	18·9
Sucrose		2	8	12	11	14	16	22·0
	Initial pH	pH						
Glycerol	5·7	6·2	6·8	7·8	8·0	8·0	8·2	
Galactose	5·2	5·3	6·1	6·9	7·5	7·5	7·4	
Glucose	5·7	6·2	7·3	8·1	8·0	8·0	8·1	
Lactose	5·2	6·5	7·0	8·0	7·2	7·0	7·8	
Sucrose	6·1	6·9	7·2	7·9	7·5	7·6	7·9	

Comparison of carbon sources. Glycerol, galactose, glucose, lactose and sucrose were compared at 10 % concentration in medium N (Table 5). On glycerol and galactose growth was slow and activity titres were correspondingly low. Growth was initially slow on lactose but eventually good mycelial felts were formed and the final activity titres were as good as those on glucose. Titres on the sucrose medium were considerably higher than on glucose, though growth was not markedly different; this result has been frequently confirmed.

Table 6. *Effect of sucrose concentration in medium N on antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani

Sucrose (%, w/v)	Initial pH	Days growth 8	12	16	20	26	30	Final dry wt. of mycelium (g.)
		Activity index						
1·0		1	1	6	7	7	4	2·9
2·5		—	5	9	10	10	10	10·1
5·0		1	5	9	10	11	12	18·5
10·0		1	7	10	11	12	13	29·6
15·0		1	9	11	12	13	14	38·0
		pH						
1·0	5·6	5·6	7·1	7·9	8·5	9·0	9·0	
2·5	5·4	5·7	7·3	7·4	7·5	8·0	8·4	
5·0	5·3	5·2	6·8	7·1	7·0	5·6	4·6	
10·0	5·4	5·8	7·0	7·6	7·5	7·4	7·1	
15·0	5·0	5·7	7·3	7·3	7·3	7·5	7·6	

An experiment (Table 6) in which sucrose concentrations were varied between 1 % and 15 % in medium N, showed the same general relationships between sugar concentration, pH drift, activity titre and weight of mycelium as was found in the experiment with glucose previously described (Table 2). It is interesting to note once more the anomalous pH drift with 5 % sugar.

Comparison of nitrogen sources. Strain 408 was grown on the simplified medium already described, containing 10 % glucose, nitrogen being supplied in various forms, namely, potassium nitrate (N), ammonium tartrate (AT), ammonium nitrate (AN), ammonium sulphate (AS) and hydrolysed casein (T). The results are presented in Table 7. Growth was very poor on AS and AN, the mycelium being almost entirely submerged. Good felts were produced on all other media, those on N being particularly good. A similar failure to grow on AS and AN media has been recorded previously (Brian, Curtis & Hemming, 1947) with the fungus *Myrothecium verrucaria* (=*Metarrhizium glutinosum*). Poor growth on these media is associated with a fall to very low pH; the degree of antifungal activity recorded in culture filtrates is remarkably high considering the poor growth. A similar unexpectedly high activity on medium AS, in spite of poor growth, is recorded in Table 8. The

Table 7. *Effect of nitrogen source in a 10 % (w/v) glucose medium on antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani

Nitrogen sources in these media are as follows: N, potassium nitrate; AN, ammonium nitrate; AS, ammonium sulphate; AT, ammonium tartrate; T, hydrolysed casein.

	Days growth							
	6	9	13	16	19	23	26	Final dry wt. of mycelium (g.)
Nitrogen source	Activity index							
N	—	—	5	6	13	14	13	16·6
AN	—	3	4	9	13	12	12	5·6
AS	—	5	9	10	13	11	9	2·1
AT	—	—	2	3	13	13	13	11·6
T	3	5	10	11	14	15	15	13·1

	Initial pH	pH						
N	5·5	5·7	6·0	6·8	7·0	7·4	7·5	7·0
AN	5·7	5·7	5·0	4·8	5·0	3·1	2·9	3·4
AS	5·7	5·6	3·8	3·2	3·0	2·8	2·8	2·8
AT	6·0	5·7	5·2	4·9	4·5	4·5	4·5	4·3
T	5·4	4·4	4·1	4·2	4·3	4·1	4·0	3·8

maximum activity reached in both these media after nineteen days was not maintained. High activity developed on media N, AT and T. No one kind of pH drift is specifically suitable for development of high titres; a steady rise in pH to about 7·5 (medium N) and a fall to pH 3·8 (medium T), were equally suitable.

In this experiment, medium T gave higher titres than any other medium but even more striking was the far more rapid establishment of a mycelial felt, which would have practical advantages in a large-scale fermentation with this somewhat slow-growing mould.

Effect of organic acid supplements in ammonia media. Brian, Curtis & Hemming (1947) showed that growth of *M. verrucaria* in ammonia media could be greatly improved by addition of various organic acids to the medium and production of glutinosin was proportionately improved. Similarly addition of various organic acids to medium AS improved growth of *A. solani* and production of the antifungal substance (Table 8).

One effect of the organic acid supplements was to prevent the rapid fall in pH which occurred in unsupplemented medium AS. The effectiveness of the acids varied from that of oxalate, which replaced the steep fall to pH 2·5 by a gradual fall to pH 4·6, to that of acetate which caused a steep rise in pH. In general terms it is true to say that the stimulating effect of the acids on growth was proportional to their effect on pH drift, acetate causing the greatest growth increases, oxalate the least. The order of antifungal activity of culture filtrates followed the same course, when only those media containing an

Table 8. *Effect of organic acid supplements to medium AS* (10 %, *w/v glucose) on antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani

All supplements were added at 1% (w/v), except acetic acid, which was added at 0·25% (w/v), before adjustment of pH to *c.* 6 with KOH.

Supplement	Days growth							Final dry wt. of mycelium (g.)
	5	9	13	16	20	23	27	
	Activity index							
—	—	1	8	10	11	10	8	3·0
Oxalic acid	—	—	1	2	3	4	5	6·3
Malonic acid	—	—	—	3	7	10	10	10·1
Citric acid	—	—	—	—	3	5	9	12·4
Malic acid	—	—	5	9	11	12	11	12·9
Fumaric acid	—	—	7	9	12	13	13	17·9
Tartaric acid	—	—	—	6	8	9	10	18·3
Succinic acid	—	—	8	12	12	13	12	19·6
Acetic acid	2	4	7	10	12	13	12	27·0

Supplement	Initial pH	pH						
—	6·0	5·9	5·0	3·0	2·9	2·9	2·7	2·6
Oxalic acid	5·7	5·6	5·6	5·1	5·2	4·8	4·6	4·6
Malonic acid	5·8	5·7	5·7	5·4	5·5	5·3	5·2	4·9
Citric acid	5·8	5·7	5·7	5·5	5·6	5·0	5·0	5·2
Malic acid	5·7	5·6	5·6	5·9	5·9	5·8	5·8	5·7
Fumaric acid	5·7	5·5	5·7	6·2	6·3	6·1	6·5	6·5
Tartaric acid	6·2	6·2	5·9	5·0	5·0	4·9	4·8	5·0
Succinic acid	5·9	5·9	5·9	6·2	6·4	6·2	6·3	6·9
Acetic acid	6·0	6·0	5·9	6·0	7·4	7·6	8·0	7·1

organic acid supplement are considered; the titres for the AS medium unsupplemented were anomalously high, in spite of very poor growth, as has already been recorded.

Further comparison of optimal media. From the series of experiments which have been described it was concluded that high titres of antifungal activity could be obtained on media containing nitrogen as nitrate, as hydrolysed casein or as ammonium sulphate in the presence of acetate, fumarate or succinate. In experiments not detailed here it was established that in each of these types of medium, high sugar concentrations were optimal and that sucrose was superior to glucose. Table 9 shows a comparison of four such media. There was little to choose between the nitrate (N), hydrolysed casein (T) and ammonium sulphate+0·25 % acetate (AS+A) media; in other experiments their relative positions might change, though the hydrolysed casein medium was perhaps most reliable owing to the rapidity with which a mycelial felt is formed. The ammonium sulphate+1 % succinate medium, in this and other experiments, was somewhat less suitable than the other three.

These three optimal media supported heavy growth and consistently high titres were obtained, although the pH drifts differed considerably. In this

Table 9. *Development of antifungal activity, pH drift and final dry weight of mycelium in cultures of* Alternaria solani *on four media (10 %, w/v sucrose) differing in nitrogen source*

Nitrogen sources in these media are as follows: N, potassium nitrate; T, hydrolysed casein; AS+A, ammonium sulphate+0·25 % (w/v) acetic acid; AS+S, ammonium sulphate+1·0 % (w/v) succinic acid.

	Days growth							
	4	8	11	16	21	25	30	Final dry wt. of mycelium (g.)
Medium	Activity index							
N	—	—	6	9	10	12	12	29·7
T	3	10	13	11	11	12	13	24·1
AS+A	—	1	8	12	13	14	15	29·1
AS+S	—	4	7	6	8	10	10	15·1

	Initial pH	pH						
N	7·0	6·9	7·3	8·4	7·8	7·9	7·9	7·1
T	6·6	6·0	5·3	4·6	5·1	4·4	4·2	4·4
AS+A	6·6	6·5	6·4	6·9	7·8	7·7	6·9	6·9
AS+S	7·0	6·8	6·3	6·8	5·3	5·2	6·3	5·6

respect the alternaric acid fermentation differs from some others. In the production of viridin by *Trichoderma viride* (Brian, Curtis, Hemming & McGowan, 1946), gladiolic acid by *Penicillium gladioli* (Brian, Curtis & Hemming, 1948) and gliotoxin by *T. viride* (Brian & Hemming, 1945), the pH drift in the culture was found to be a main factor influencing production and accumulation of the antibiotic. In the production of glutinosin by *M. verrucaria* (Brian, Curtis & Hemming, 1947) and griseofulvin by *P. janczewskii* (Brian, Curtis & Hemming, 1946), there was no relation between pH drift and production of the antibiotic, but neither was there any obvious relation between yield of mycelium and yield of antibiotic. In production of alternaric acid by *A. solani*, high titres are almost always correlated with high yield of mycelium. Inspection of the data presented in Tables 2–9 will confirm this, the only significant exceptions being the somewhat high but transient titres recorded on ammonium nitrate (AN) or ammonium sulphate (AS) media in spite of poor growth.

Isolation of alternaric acid

Isolation of the active material has only been attempted with two strains of *A. solani*—Nos. 407 and 408. Much the heavier yields have been obtained with 408 and this strain is now used exclusively for production. The substance isolated is the same from both strains.

Development of a method of extraction and purification proceeded simultaneously with the work on development of optimal culture media. In the early stages of the investigation, culture filtrates of low activity only were available. Under those conditions a method of extraction was developed which proved to be ineffective for culture filtrates of higher activity; conversely, the method adopted finally for highly-active filtrates had been found ineffective for the earlier batches of low activity. As this relation between isolation technique and concentration of the active material in the fermentation liquor is of some interest, a short account of the development of the extraction methods is given below.

Culture filtrates of three different levels of activity became available during the development process: (*a*) from strain 407 on glucose+nitrate media, of low activity; (*b*) from strain 408 on glucose+nitrate media, of moderate activity; (*c*) from strain 408 on sucrose+nitrate media, of high activity. There were three corresponding stages in the development of the isolation techniques.

Process 1. It was found that the active material could be removed from culture filtrates adjusted to pH 3·5 by extraction with chloroform, benzene or *n*-butanol. Extraction at pH 5·5 or above was inefficient. On evaporation of these solvents, a brown gummy material was produced which could not be recrystallized satisfactorily from petroleum ether, carbon tetrachloride or water; the gum was highly soluble in all other solvents. The chloroform extract was therefore shaken three times with equal volumes of a pH 5·5 buffer and most of the activity was transferred to the buffer. Experience showed that extraction of chloroform extracts with buffer was best done in an atmosphere of nitrogen, otherwise there were considerable losses of activity. When the buffer was then acidified to pH 3·5 and extracted with an equal volume of carbon tetrachloride, 75 % of the activity was removed from the buffer. Much coloured impurity was removed by this process; carbon tetrachloride effected more purification than chloroform. On evaporating the carbon tetrachloride a pale yellow gum was obtained. On recrystallization from water, colourless plates were obtained, causing marked stunting of *B. allii* germ-tubes at 0·1 μg./ml. or even lower concentrations. This substance was found (Brian *et al.*, 1949) to be an optically active dibasic acid and was named alternaric acid. Yields of the order of 1–2 mg./l. were obtained from low-activity culture filtrates in this manner.

Process 2. Using the more active glucose+nitrate culture filtrates from strain 408, which also produced much less pigment, it was found possible to obtain a crystalline product directly from the gummy material obtained by evaporation of the first chloroform extract. It was difficult to remove all brown colour and the procedure, outlined above as process 1, was still used, with the modification of decreasing the volume of the first chloroform extract before re-extracting into buffer. Yields of the order 5–10 mg./l. were obtained.

Process 3. When highly-active culture filtrates from strain 408 on sucrose+nitrate media (7·5 % sucrose) became available it was found that the extraction processes described above were unnecessarily complicated. The previously rejected method of evaporation of the first chloroform extract, followed by recrystallization, was again tried and, under these conditions, found successful. The method now adopted is to adjust culture filtrates to pH 3·5, extract twice with 1/10 vol. of chloroform, evaporate the chloroform extract to dryness under reduced pressure and recrystallize from benzene. Yields of alternaric acid, under these conditions, are high; the mean yield of ten batches was 140 mg./l., the highest yield in the series being 236 mg./l.

It has been shown earlier that a casein-hydrolysate medium (T) is frequently superior to the nitrate medium (N). These two media, both containing 7·5 % sucrose, were compared on three occasions under comparable conditions. The mean yields were: T, 167 mg./l.; N, 119 mg./l. The superiority of medium T was thus confirmed.

Medium AS, which has earlier been shown to give unexpectedly high assays, gave a mean yield, in comparable experiments, of only 22 mg./l. The high titres observed still require explanation.

Antibiotic activity of alternaric acid

Experiments to determine the stability of aqueous solutions of alternaric acid showed that solutions in Czapek-Dox solution could be boiled for 5 min. in the range pH 3–7·6 without loss of activity and could be autoclaved (15 lb./sq. in.) for 20 min. without loss of activity in the range pH 4–7·6, but that autoclaving at pH 3 caused a partial loss of activity.

No antibacterial activity has been noticed; the following organisms grew normally in broth containing 100 μg. alternaric acid/ml.: *Bacillus lactis aerogenes, B. brevis, B. subtilis, Staph. aureus, S. typhi* and *Bact. coli* (two strains).

Antifungal activity was tested by a spore germination test at pH 3·5. Table 10 shows the least concentrations (*a*) inhibiting germination, and (*b*) causing stunted germ-tubes, of a number of fungi. The extreme specificity should be noted; some fungi, e.g. *M. verrucaria*, are extremely sensitive, germination being completely inhibited by low concentrations of alternaric

Table 10. *Antifungal activity of alternaric acid at pH* 3·5

Fungus	Least concentration (μg./ml.) of alternaric acid: Inhibiting germination	Least concentration (μg./ml.) of alternaric acid: Causing stunting
Absidia glauca Hagem	0·8	0·1
Aspergillus niger van Tiegh.	100·0	25·0
A. tamarii Kita	50·0	3·1
Botrytis allii Munn.	>100·0	0·01
Fusarium caeruleum (Lib.) Sacc.	>100·0	100·0
F. graminearum Schwabe	>100·0	25·0
Myrothecium verrucaria (Alb. and Schw.) Ditm. ex Fries	0·1	0·01
Mucor mucedo Bref.	0·4	0·01
Penicillium brevicompactum Dierckx	>100·0	50·0
P. digitatum Sacc.	>100·0	12·5
P. expansum Link	100·0	12·5
P. gladioli Machacek	50·0	25·0
Stemphylium sp.	25·0	0·04
Thamnidium elegans Link	0·8	0·005

acid; others, e.g. *Fusarium caeruleum*, are very resistant, high concentrations having no noticeable effect; still others, e.g. *B. allii*, show stunted germ-tubes at low concentrations, though much higher concentrations fail to prevent germination.

As in most cases where a toxic agent is a weak acid, alternaric acid is antifungal only in the undissociated state; at pH 6·5 a concentration of 100 μg./ml. is without visible effect on *B. allii* or *M. verrucaria*.

The stunting effect on fungal hyphae might be produced in several ways, which could not be distinguished by an assay where one observation is made at a fixed time-interval after setting-up the assay. There are three main possibilities: (*a*) an extension of the latent period before germination commences, followed by normal germination and growth; to produce the observed effects this would mean an extension of the latent period from 1·5 hr. to at least 14 hr.; (*b*) a diminution of the rate of hyphal extension after germination and formation of germ-tubes has taken place; (*c*) a general slowing-down of all the processes of germination and hyphal extension. Several experiments have shown that the second of these possibilities is, in fact, what takes place.

Table 11. *Germination and germ-tube extension of* Botrytis allii *in the presence of alternaric acid, at 25°*

Time (hr.)	Alternaric acid (μg./ml.)					
	0	1	10	0	1	10
	Germination (%)			Mean germ-tube length (μ)		
3	14	23	18	0·9	1·3	1·2
4·25	26	44	20	2·0	3·8	2·0
5·5	39	53	37	3·0	5·0	3·6
7·5	63	53	43	7·2	5·7	4·4
10·5	90	68	70	54·8	11·0	.
12·25	100	70	60	82·2	10·8	9·0
24	100	76	68	462·0	12·6	10·2

The results of periodical counts of percentage germination and measurements of germ-tube length of *B. allii* spores, in solutions of alternaric acid and in normal nutrient, are shown in Table 11. The final effect of 1 μg. alternaric acid/ml., after 24 hr., is to decrease percentage germination by 14% only but to decrease mean germ-tube length from 462 μ. to 12·6 μ. Using a stronger solution (10 μg./ml.) the effects are slightly more marked. In spite of this marked final effect, for about 6·5 hr. after setting-up the experiment, or about 5 hr. after the commencement of germination, alternaric acid has little or no effect; after that point, while the germ-tubes in the normal medium elongate exponentially at a rapid rate, the increase in length of germ-tubes in alternaric acid is very slow. The stunting of germ-tubes caused by alternaric acid is therefore produced by a decrease in the rate of elongation of the young hyphae which does not take effect until some hours after germination has commenced. The further analysis of this interesting effect will be dealt with in a subsequent publication.

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Requirements for Pyocyanine Production by *Pseudomonas aeruginosa* (Schroeter) Migula

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SUMMARY: Substances found to be essential for pyocyanine production by *Pseudomonas aeruginosa* were Mg, PO_4, SO_4 and NH_4 ions, together with a carbon source, preferably glucose, glycerol or ethanol. Ca, Na, K, Fe ions were not essential, nor was asparagine. Pyocyanine was formed in nutrient broth prepared with distilled water; the addition of chalk improved pigment production. Reliable and good yields were regularly obtained on an ordinary broth medium containing glucose (0·2 %) and chalk.

Recommended media for stimulating pyocyanine production were tested. Turfitt's medium gave more reliable pyocyanine formation when the NH_4NO_3 was replaced by NH_4Cl and chalk was added. Increasing the $MgSO_4 . 7H_2O$ content of this medium from 0·025 % to 0·2 % inhibited pyocyanine production.

Various amino-acids were tested. Glycine, L-tyrosine, DL-alanine, L-leucine, when added separately or in various combinations to a salt mixture + glucose, glycerol or ethanol permitted the production of pyocyanine. A mixture of glycine, L-leucine and DL-alanine (recommended by Burton, Campbell & Eagles, 1947) exerted a stimulating effect on pyocyanine production far superior to that obtained on any other medium tested.

The highest yield reached was 23·1 mg. pyocyanine/100 ml. of a medium consisting of: 0·4 % DL-alanine; 0·8 % L-leucine; 0·2 % $MgSO_4 . 7H_2O$; 0·05 % K_2HPO_4; 0·001 % $FeSO_4 . 7H_2O$; 1 % glycerol and chalk.

There is agreement in the literature that sulphate, phosphate and magnesium are essential for pyocyanine production by *Pseudomonas aeruginosa* (*Ps. pyocyanea*). Jamieson (1942) reported that, by chance using tap water of lake origin for his media, the majority of certain organisms he had previously considered to be *Achromobacter* spp. produced blue-green pigment; this would place them as *Pseudomonas* spp. Even media such as proteose-peptone when prepared with distilled water, failed to yield pigment, but with tap water did so. Jamieson found that best pigmentation was produced on Sullivan's medium: asparagine 1 %, magnesium sulphate 0·2 %; dipotassium phosphate 0·1 %; distilled water. He thought this was due primarily to asparagine being a source of nitrogen more adequate for pigment formation than peptone. Burton, Campbell & Eagles (1948) found that K and Fe ions in addition to SO_4, PO_4, and Mg ions were necessary. They stated that Na, NH_4, Mn, Zn, AsO_4 or Cu could not be substituted in their defined medium (containing also 1 % glycerol, 0·6 % glycine, 0·6 % leucine) for the above ions, and that NH_4, Mn, Zn, AsO_4 and Cu ions inhibited pyocyanine production. On the other hand Liot (1923) found that ammonium ion (as an organic salt) was necessary for pyocyanine formation. Young (1947) found that no pigment was produced in media containing over 1 % glucose, the inhibition being attributed to the attainment of low pH values. Turfitt (1937) recommended the following medium for good pigmentation: NH_4NO_3, 0·1 %; K_2HPO_4, 0·025 %; $MgSO_4 . 7H_2O$, 0·025 %; ethanol, 0·1 %; distilled water.

A strain of *Ps. aeruginosa* isolated by the author from the River Yarkon, Israel, showed no pigment production under anaerobic conditions, but produced good pigmentation when grown aerobically on nutrient broth or nutrient agar prepared with distilled water. Because of this difference from Jamieson's findings, the requirements of our strain of *Ps. aeruginosa* for pyocyanine production were examined.

METHODS AND MEDIA

Method of estimating pyocyanine. The pyocyanine was extracted from culture media and the colour intensity of extracts was compared with those of known concentrations of pure pyocyanine in chloroform with a photoelectric colorimeter (Lumetron Electrophotometer; scale standardized at 100 with pure chloroform, colour filter 620, light intensity +10). The logarithms of the Lumetron readings when plotted against pyocyanine concentration gave a straight line relationship.

Growth and extraction of pyocyanine. Twenty ml. quantities of medium in 50 ml. round, flat-bottom flasks were sterilized at 110° for 45 min. The pH after sterilizing was determined on separate 5 ml. quantities. Each flask was inoculated with one loopful of a young active culture and incubated at 37°. In the first experiments, one set of flasks was examined for pyocyanine on the 7th or 8th day, and a further set after a longer incubation period. In later experiments pyocyanine extractions were carried out on the 4th and 7th/8th day. In practically all cases pigment production decreased on prolonging the incubation beyond 4–5 days.

Table 1. *The composition of various media used to ascertain requirements of* Ps. aeruginosa *for pyocyanine production*

	Concentration	Medium number													
Constituent	(%)	1	2	3	4	5	6	7	8	9	10	11	12	16	17
$MgSO_4 . 7H_2O$	0·02	−	+	+	+	−	+	+	+	+	+	−	+	−	+
$CaCl_2$	0·01	+	−	+	+	+	+	+	+	+	+	−	−	−	−
NaCl	0·1	+	+	−	+	+	+	+	+	+	+	−	−	−	−
KH_2PO_4	0·1	+	+	+	−	+	+	+	−	+	+	+	+	−	−
K_2HPO_4	0·1	−	−	−	−	−	−	−	−	−	−	+	+	+	+
$MgCl_2$	0·02	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Na_3PO_4	0·2	−	−	−	−	−	−	−	+	−	−	−	−	−	−
$NaHCO_3$	0·1	−	−	−	+	−	−	−	−	−	−	−	−	−	−
$FeCl_3$	0·001	−	+	+	+	+	+	−	+	+	+	−	−	−	−
$FeSO_4 . 7H_2O$	0·001	+	−	−	−	−	−	−	−	−	−	−	−	−	−
KNO_3	0·1	−	−	−	+	−	+	−	−	−	−	−	−	−	−
NH_4NO_3	0·1	+	+	+	+	+	−	+	+	+	+	−	−	−	+
Glycerol	2·0	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Asparagine	0·05	+	+	+	+	+	+	+	+	+	−	+	+	+	+

+ = present in medium; − = absent from medium.

The individual cultures were extracted with 4–5 successive portions (5 ml.) of chloroform. Generally the chloroform extract was turbid; it was easily cleared by a brief centrifugation. To ensure perfect clarity for colorimetric measurements it was filtered through a filter paper wetted with chloroform, and the volume made up the original volume of the culture. When too concentrated the solution was suitably diluted.

Media. The composition of fourteen media is given in Table 1. In addition the two media recommended by Turfitt (1937) for stimulating the green fluorescent pigment of *Ps. fluorescens* (0·05 % $MgSO_4 . 7H_2O$; 0·05 % K_2HPO_4; 0·3 % asparagine) and pyocyanine production by *Ps. pyocyanea* (0·025 % $MgSO_4 . 7H_2O$; 0·025 % K_2HPO_4; 0·1 % NH_4NO_3; 0·1 % ethanol) were tested (media 18 and 19 respectively). Other media tested were: standard broth containing 0·3 % Difco meat extract+0·5 % Bacto Proteose peptone made up with distilled water (No. 13); the same+0·2 % glucose (No. 14); standard broth+0·2 % glucose+chalk (No. 15).

RESULTS

In the first series of experiments (Table 2) growth occurred in all media except that containing no phosphate (No. 4). The defined media Nos. 1 to 10, when lacking Mg, SO_4, PO_4, NH_4 or glycerol, failed to produce pyocyanine. Ca,

Table 2. *Effect of composition of medium on growth, pH and pyocyanine production by* Ps. aeruginosa *grown at 37°*

		pH values					
		Initial Series		Final Series		Pyocyanine mg./100 ml. medium	
Medium No.	Growth	1	2	1	2	1*	2**
1	moderate	6·4	6·4	7·3	8·1	None	None
2	good	6·3	6·4	6·1	6·4	2·45	0·35
3	good	6·4	6·4	6·2	6·3	2·24	0·28
4	none	6·7	6·4	6·7	7·6	None	None
5	good	6·3	6·3	7·5	7·0	Traces	None
6	good	6·4	6·5	6·8	7·5	None	None
7	good	6·3	6·4	6·3	6·2	1·87	0·1
8	good	7·8	6·9	7·4	6·8	1·79	0·1
9	good	6·3	6·9	6·5	7·3	None	None
10	good	6·3	6·4	6·1	5·7	1·72	0·15
11	moderate	6·6	—	6·5	—	None	.
12	moderate	6·5	—	4·9	—	None	.
13	very good	6·5	6·9	8·8	8·7	0·4	0·18
					8·7		1·37+
14	very good	6·4	6·7	7·3	7·4	1·62	0·29
15	very good	7·4	7·4	8·6	8·7	5·62	12·63
					8·7		9·38+
16	fair	7·7	—	6·5	—	None	.
17	good	7·5	—	4·9	—	0·15	.
18	good	7·7	7·0	7·8	8·5	None	None
19	good	7·8	7·0	7·2	4·2	1·42	None

*=Series 1 tested on 7th day; **=Series 2 tested on 8th day; +=chalk added to medium.

Na, K, Fe ions and asparagine were not essential for pigment formation. Substitution of KNO_3 for NH_4NO_3 (No. 6) did not stimulate pyocyanine formation, indicating that the ammonium ion was required and not nitrate ion. In medium No. 12 (a defined medium containing Mg, SO_4, PO_4, asparagine and glycerol, but no NH_4 ion) pigment was produced, confirming the need for ammonium nitrogen. Turfitt's medium for *Ps. fluorescens* lacking in glycerol and ammonium salts but containing the essential ions Mg, SO_4, PO_4, with asparagine as nitrogen source (No. 18), also did not yield pyocyanine. In Turfitt's medium for *Ps. aeruginosa* containing Mg, SO_4, PO_4, and NH_4 ions, and ethanol instead of glycerol (No. 19), pyocyanine was formed but only after four days. Medium No. 17 which contained the substances which appeared to be essential for pigment formation (Mg, SO_4, PO_4, NH_4 ions and glycerol) gave at first a faint greenish pigmentation, which turned pink and became reddish by the fourth day, presumably due to the low pH reached; the acid salt of pyocyanine is port-wine coloured. On making alkaline a little pyocyanine was extractable from these cultures.

The best production of pyocyanine occurred on standard broth containing glucose (0·2 %)+chalk (Medium 15): yields as high as 12·6 mg. pyocyanine/100 ml. medium were obtained. On unsupplemented standard broth pigmentation was erratic; some pyocyanine was formed when 0·2 % glucose (without chalk) was added.

Ammonia, which appeared to be essential for pigment formation, is liberated by the organism from the peptone in standard broth. Turfitt's relatively simple medium (No. 19) contains NH_4NO_3, but this medium was unreliable for pyocyanine production; fluorescin, which is also produced, was at times the sole pigment present. Trials were made substituting NH_4Cl for the NH_4NO_3 in this medium and doubling the ethanol content (i.e. to 0·2 %); the pH of all media was adjusted to 7·4. In one medium of this series, ethanol was replaced by glycerol, and asparagine used instead of ammonium salt; growth was good, the pH of the medium rose to 8, fluorescin but no pyocyanine was formed. Pyocyanine formation was erratic on Turfitt's medium both with and without chalk. With NH_4Cl growth was fair to moderate, the pH of the culture medium always fell to 4·4–5·2, but no pyocyanine was formed; with the addition of chalk, the pH rose to 8·1 and pyocyanine was regularly formed although in variable amounts, the highest being 3·3 mg./100ml. medium. In general NH_4Cl with chalk was more reliable for pyocyanine formation and gave higher yields than NH_4NO_3. These chemically defined media were not as good for pyocyanine production as standard broth containing glucose and chalk.

L-Tyrosine, L-tryptophan, L-cystine and glycine were added separately and in various combinations to 0·2 % glucose, 2 % glycerol or 0·2 % ethanol; the salts present were: 0·025 % $MgSO_4 . 7H_2O$; 0·025 % K_2HPO_4; 1 % NH_4NO_3 or NH_4Cl. The media were adjusted to pH 7·4 and cultures incubated for four days at 37°. All cultures showed good growth, the final pH varying from 7·6 to 8·7. Pyocyanine was produced only in the media containing tyrosine or glycine. Yields from glycine media were distinctly better than from tyrosine media; the presence of chalk increased these yields, although the effect was apparently not due to control of pH. There were no striking differences between media containing either glucose, ethanol or glycerol. A comparison of pyocyanine yields from media containing 0·2 % glycine+ammonium salts, with those from media lacking glycine, gave results somewhat in favour of the latter.

The two media of Burton, Eagles & Campbell (1947) and recommended by them for maximum production of pyocyanine were compared with the standard broth containing glucose and chalk. One of these media contained 0·4 % glycine, the second 0·4 % DL-alanine; both were at pH 7·4 and contained also 0·8 % L-leucine, 1 % glycerol and a salt mixture ($MgSO_4 . 7H_2O$, 0·2 %; K_2HPO_4, 0·04 %; $FeSO_4 . 7H_2O$, 0·001 %). Pyocyanine extractions were carried out after four days at 37°. The results (Table 3) with both media of Burton *et al.* were striking; they gave yields of pyocyanine far superior to the supplemented standard broth. The addition of chalk to the media of Burton *et al.* tended further to increase the pyocyanine yield.

Table 3. *Effect on pyocyanine production of L-leucine, with glycine or DL-alanine in a glycerol+salt mixture,* as compared with standard broth+glucose*

	0·4 % glycine+ 0·8 % L-leucine		0·4 % DL-alanine+ 0·8 % L-leucine		Standard broth+ 0·2 % glucose	
	$-CaCO_3$	$+CaCO_3$	$-CaCO_3$	$+CaCO_3$	$-CaCO_3$	$+CaCO_3$
Final pH	8·0	7·7	8·1	7·9	8·6	8·7
Pyocyanine (mg./100 ml. medium)	14·5	19·5	19·5	23·1	7·3	9·15

* 1 % glycerol; 0·04 % K_2HPO_4; 0·2 % $MgSO_4 . 7H_2O$; 0·001 % $FeSO_4 . 7H_2O$.

Table 4. *Pyocyanine production on various media*

(The growth was very good in all media.)

No.	Medium	$CaCO_3$	Final pH value	Pyocyanine (mg./100 ml. medium)
1	$MgSO_4 . 7H_2O$ 0·2 % K_2HPO_4 0·04 % NH_4Cl 0·1 % Ethanol 0·2 %	−	4·6	(trace)
		+	7·3	(trace)
2	$MgSO_4 . 7H_2O$ 0·2 % K_2HPO_4 0·04 % Glycine 0·4 % L-Leucine 0·8 % Ethanol 0·2 %	−	8·3	6·85
		+	8·6	4·4
3	As No. 2+ $FeSO_4 . 7H_2O$ (0·001 %)	−	8·6	5·46
		+	8·4	5·73
4	As No. 3 but with glycerol (1 %) replacing ethanol	−	6·9	10·44
		+	6·9	10·44
5	Standard broth + glucose (0·2 %)	−	8·6	3·58
		+	8·4	7·19

In further tests with the media of Burton *et al.* it was found (Table 4) (*a*) that although comparatively good yields of pyocyanine were obtained with ethanol, glycerol was better; (*b*) ferrous sulphate did not appear to exert an influence on pyocyanine formation; (*c*) increasing the concentration of $MgSO_4 . 7H_2O$ from 0·025 % to 0·2 % inhibited pyocyanine formation in the given medium.

DISCUSSION

The results obtained with our strain of *Ps. aeruginosa* do not agree with Jamieson's (1942) finding that tap water was essential for pyocyanine formation on nutrient broth; pyocyanine was obtained in nutrient broth cultures prepared with distilled water, but the addition of calcium carbonate did enhance its formation. Wrede & Strack (1924) found that media containing asparagine+glucose as carbon source gave good pyocyanine yields, and recognized the importance of maintaining the organism in a medium containing peptone (1 %) to preserve its ability to give good yields of pigment. In the present experiments asparagine was not essential for pyocyanine formation; when it was added to a medium containing glycerol, $MgSO_4$ and K_2HPO_4 no pyocyanine was formed, yet on replacing asparagine with an ammonium salt pyocyanine was formed. Peptone is favourable for the maintenance of the organism's ability to give good yields, but a peptone medium did not ensure high pyocyanine yields unless the medium also contained glucose and chalk. We obtained pyocyanine in nutrient media+2 % glucose, whereas Young (1947) found no pigment in media containing over 1 % glucose.

The present findings do not agree with those of Burton *et al.* (1948) that K and Fe are essential ions for pyocyanine formation. The media used without added iron contained less than 0·02 p.p.m. iron and yet pyocyanine was formed.

Ammonium ion appeared essential for pigment formation and we do not agree with Liot (1923) who claimed that pyocyanine is formed only in the presence of an organic ammonium salt. Ammonium salts as sole nitrogen source in media containing 0·025 % $MgSO_4 . 7H_2O$, 0·025 % K_2HPO_4, with glucose, glycerol or ethanol gave somewhat better yields than when glycine was added to the medium, whereas in media containing 0·2 % $MgSO_4 . 7H_2O$ and 0·04 % K_2HPO_4 pyocyanine production was inhibited. Turfitt's medium for pyocyanine formation was found to be unreliable, but by replacing ammonium nitrate with ammonium chloride and chalk the pigment was regularly formed.

The media devised by Burton *et al.* (1948) were superior in supporting pyocyanine production to all the other media tested with a particular strain of *Ps. aeruginosa* in the present work.

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Extraction of the T Antigen of *Streptococcus pyogenes*

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SUMMARY: The heat-stability of the T antigen of strains of *Streptococcus pyogenes* grown in Pope & Smith (1932) broth (containing maltose and yeast) with added pancreatic extract varied considerably from strain to strain; with several strains 30 minutes' boiling was insufficient to eliminate all T agglutinability.

By heating suspensions of streptococci to 80° for 30 min. and then treating the heated suspensions with trypsin-containing pancreatic extract for a few hours, T antigen was obtained in solution and could be demonstrated by precipitin tests with appropriate type-specific sera.

It is well known that streptococci of Lancefield's Group A (*Streptococcus pyogenes*) have two type-specific antigens, known as M and T (Lancefield, 1940–1). Both these antigens may be concerned in type identification by the slide agglutination method but the suspensions of streptococci that have to be used for this test are frequently unstable and the method commonly used for stabilizing them comprises digestion with trypsin—a process that destroys the M but not the T antigen. Consequently, in practical slide agglutination typing, one often has to rely on the T antigen alone, which may be confusing owing to the sharing of T antigens by streptococci of different types (Stewart, Lancefield, Wilson & Swift, 1944). For the precise study of the T antigen content of streptococci it would therefore be useful to be able to destroy this antigen in the cells without affecting the M antigen.

It would also be of great value if the T antigen could be extracted from the cocci and demonstrated by a precipitin method similar to that used for the M antigen. Elliott (1943) and Lancefield & Dole (1946) selectively destroyed the T antigen by heating the streptococcal suspension to 100° for 30 min.; and Lancefield & Dole demonstrated that a T-containing extract could be obtained by prolonged pepsin or trypsin digestion of the cocci. The present paper reports some extensions of this work.

EXPERIMENTAL

The antisera used were prepared at various times in this laboratory, usually by repeated intravenous inoculation of formalin-killed streptococci into rabbits, and were absorbed with heterologous strains to remove Group antibodies. The strains of streptococci used were all 'classical' strains, stored in the laboratory in the dried state. Agglutination tests were carried out by the slide technique, and precipitin tests by the method devised by Swift, Wilson & Lancefield (1943) for M precipitin tests.

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Destruction of T antigen by heat

It should be noted first that all the experiments reported here refer to loss of ability to agglutinate with T antisera, and that this is not necessarily the same as destruction of the T antigen. Testing for heat destruction of the T antigen by slide agglutination is often difficult because heated suspensions are very commonly unstable. It was found, however, that the digest broth described by Pope & Smith (1943), to which had been added 1% (v/v) pancreatic extract (containing about 50 standard units of trypsin/ml.), generally gave suspensions that were still satisfactory even after prolonged heating at 100°. Strains of Types **4**, **24**, **26**, **29**, and **46**, which are known to share a common T antigen (Stewart *et al.*, 1944) were grown in this medium

Table 1. *Heat sensitivity of T antigen shown by slide agglutination tests*

Strain	Time of boiling (min.)	Serum **4** (*T*) 'Wheatley' diluted						
		1/20	1/40	1/80	1/160	1/320	1/640	1/1280
		Agglutination result						
4 'T4' *T*	0	+++	+++	+++	+++	+++	+++	+++
	5	+++	+++	+++	+++	+++	++	++
	10	+++	+++	+++	+++	+++	++	+
	20	+++	+++	+++	++	+	+	–
	30	+++	+++	++	++	+	–	–
	40	+++	+++	++	++	–	–	–
24 'C115' *M* & *T*	0	+++	+++	+++	+++	+++	+++	++
	5	+++	+++	+++	+++	++	±	–
	10	+++	+++	+++	++	++	–	–
	20	+	+	+	+	–	–	–
	30	+	+	+	–	–	–	–
	40	–	–	–	–	–	–	–
26 'glossy' *M* & *T*	0	+++	+++	+++	+++	+++	+++	++
	5	++	++	++	++	+	–	–
	10	+	+	+	+	–	–	–
	20	+	+	+	±	–	–	–
	30	–	–	–	–	–	–	–
29 'D23' *M* & *T*	0	+++	+++	+++	+++	++	–	–
	5	–	–	–	–	–	–	–
46 'C105' *M* & *T*	0	+++	+++	+++	+++	+++	+++	++
	5	+++	+++	+++	+++	++	+	–
	10	++	++	++	++	+	–	–
	20	++	++	++	+	+	–	–
	30	+	+	+	–	–	–	–
	40	+	+	–	–	–	–	–

Decreasing strength of agglutination is indicated by the symbols +++, ++, +, and ±, respectively; – = no agglutination.

In this and all subsequent tables, the following conventions are adopted:

Numbers in bold type, e.g. **4** = Type number.

Numbers in quotation e.g. 'T4' = strain number or name.

Letters in italic, e.g. *M* = antigen content of strain, or antibody content of serum.

Table 2. *Heat sensitivity of T antigen shown by slide agglutination test*

Type: Strain:	Richards 'GIII' *M & T*			13 'Glover' *T*			15 'T15/13/0' *M & T*			27 'SF40' *T*			
Time of boiling bacterial suspensions (min.) Sera (diluted 1/5)...	Richards 'GIII' *M & T*	13 *T*	B3264 *T*	Richards 'GIII' *M & T*	13 *T*	B3264 *T*	15 *M & T*	17 *M & T*	23 *M & T*	11 *M & T*	12 *M & T*	27 *T*	44 *M & T*
0	+++	+++	+++	+++	+++	+++	+++	++	+++	+	+	+++	+++
5	+++	++	++	+	+	—	+++	++	++	+	+	++	++
10	+++	++	++	+	+	—	++	++	++	+	—	++	++
15	++	+	—	—	—	—	++	+	++	+	—	++	++
25	+	—	—	.	.	.	++	—	+	+	—	+	+
35	.	.	.	.	.	.	.	granular	.	+	—	+	+
45	.	.	.	.	.	.	.	.	.	+	—	+	+

. = not tested. Other conventions as in Table 1.

for 18–24 hr. at 37°; small samples of the cultures were boiled for varying times and then tested with dilutions of a Type 4 antiserum. Control tests were made with saline and with heterologous type-specific sera, and were in all cases negative. There was considerable variation in the duration of heating necessary to eliminate the T-agglutination (Table 1), and in three cases agglutination still occurred after 30 min. heating. Similar tests were carried out with other types, but in these cases the sera were used at one dilution only (1/5). The sera used included those of heterologous types thought to share a T antigen with the strain under test, because any agglutination thus observed would almost certainly be due to the shared T antigen and could not be due to the heat-stable M antigen. There was a similar variation in the time taken for loss of T agglutination (Table 2), perhaps partly to be explained by variations in the initial antibody titre of the serum, and in one case 30 min. at 100° was insufficient for complete destruction.

It seemed clear therefore that under our conditions, and with Pope & Smith broth containing pancreatic extract, 30 min. boiling could not, with all strains, be relied on to eliminate agglutination due to the T antigen. This differed from the published experience of other workers. The discrepancy is probably attributable to differences in culture media since in a number of experiments with strains of Types 4 and 8 grown in a meat-infusion nutrient broth enriched with 0·5 % glucose, 5 min. boiling usually proved sufficient to render the streptococci inagglutinable by T antisera. A typical result is shown in Table 3. This increased heat-lability was not, however, constant with different batches of broth and clearly requires much more investigation; it might be due to pH differences, since Lancefield & Dole (1946) found a decreased pH to aid heat destruction of isolated T antigen, although in the present work the cocci were always washed well before testing.

Table 3. *Loss of T agglutinability on heating by streptococci grown in nutrient broth with* 0·5 % *glucose*

		Serum 26, *T* only								Serum 26, *M*
Strains	Suspension	Undiluted	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	(undil.)
4 *T*	1	+++	+++	+++	+++	+++	++	++	–	–
	2	–	–	–	–	–	–	–	–	–
26 *M* & *T*	1	+++	+++	++	++	++	±	–	–	+++
	2	–	–	–	–	–	–	–	–	+++

Suspension 1—bacteria grown in Pope & Smith broth, washed and boiled 5 min.

Suspension 2—bacteria grown in nutrient broth with 0·5 % glucose, washed and boiled 5 min.

Other conventions as in Table 1.

It was then found that specific T agglutination of cocci grown in Pope & Smith broth was lost after only a short period of heating when the cocci were first treated with pancreatic extract for about 1·5 hr. The loss of the T agglutination could also be achieved by treatment with pancreatic extract for 1·5 hr. subsequent to 10 min. boiling (Table 4). Lancefield & Dole showed, and I have confirmed, that such treatment itself produces no demonstrable effect on specific agglutination by T antisera.

Table 4. *Loss of T agglutinability after boiling and trypsin digestion*

		Sera			
		4 'Wheatley' *T*	**24** 'G54' *T*	**26** 'Glossy' *T*	**46** 'C105' *M & T*
Strains	Suspension	Agglutination result			
4, 'T4' *T*	1	+++	+++	+++	+++
	2	±	−	−	−
24, 'C115' *M & T*	1	+++	+++	+++	+++
	2	−	−	−	−
26, 'Glossy' *M & T*	1	+++	+++	+++	+++
	2	−	−	−	−
28, 'D140A' *M & T*	1	+++	++	++	++
	2	++	−	−	−
29, 'D23' *M & T*	1	±	−	−	±
	2	−	−	−	−
46, 'C105' *M & T*	1	+++	+++	+++	+++
	2	−	−	−	−

Suspension 1 boiled for 10 min.

Suspension 2 boiled for 10 min. and subsequently treated with pancreatic extract for 1·5 hr.

Other conventions as in Table 1.

After treatment by boiling and pancreatic extract all the cross-reactions between strains and sera of Types 4, 24, 26, 28, 29 and 46 which were attributable to the common T antigen were lost. There remained a cross-reaction between Types 4 and 28; but it will be remembered that the M antigen of Type 28, unlike those of other types, is not trypsin-sensitive and it is just possible that this cross-reaction resulted from the presence in the Type 4 serum of some antibody to this antigen of Type 28. Rarely, when the strains were treated with pancreatic extract before boiling, they showed minor cross-reactions; but more often all agglutinability was lost as when the treatment was carried out after boiling.

Extraction of T antigen

Lancefield & Dole (1946) obtained enough T antigen for precipitin tests in the supernatant fluids of 'trypsinized' suspensions with only one type, namely, Type 1; for other types the extracts had to be concentrated from much larger quantities of culture. In their hands the T antigen appeared to be rapidly destroyed by heating. The ease with which, in our experiments, heated cocci lost their T antigen when treated with pancreatic extract suggested that the antigen might be liberated into the supernatant fluid rather than destroyed by this combination of treatments. Cultures of strains of Types **4**, **24**, **26**, and **46** grown in 1 litre volumes of buffered glucose broth (modified from Todd & Hewitt, 1932) at 37° overnight, were centrifuged, washed several times and resuspended in 2·7 ml. saline; the suspension was boiled for 5 min. and cooled; 0·3 ml. of pancreatic extract was then added and the pH brought to 8·2. After 4 hr. digestion at 37° the supernatant fluids from the centrifuged suspensions were tested for precipitinogen with sera of Types **4** and **46** known to contain T antibodies for all the strains tested. However, only the extracts from the Type **4** and **46** strains gave precipitates with the Type 4 antiserum and none precipitated with the Type **46** serum. Following the method of Lancefield & Dole the extracts were brought to pH 2·5; the resulting precipitates were separated and dissolved in 0·5 ml. of pH 7·8 phosphate buffer. The Type **4** and **46** extracts concentrated in this way both gave precipitates with Type **4** and **46** antisera, but Types **24** and **26** still gave no precipitates. Extracts containing T antigens reacting with homologous sera were, however, prepared by this method from strains of Types **1**, **2**, and **25**, but not from strains of Types **12** and **22**.

Table 5. *Loss of T agglutinability by heating and treatment with pancreatic extract*

		Serum dilutions*						
		1/20	1/40	1/80	1/160	1/320	1/640	1/1280
		Agglutination result						
Strains	Suspension							
4	1	+++	+++	+++	+++	+++	+++	++
'T4'	2	+++	+++	+++	++	++	++	+
T	3	+	+	+	+	+	+	−
27	1	+++	+++	+++	+++	++	+	−
'SF40'	2	+++	+++	+++	+++	++	−	−
T	3	++	++	++	+	+	−	−
46	1	+++	+++	+++	+++	+++	++	+
'C105'	2	+++	+++	+++	++	+	+	−
M & T	3	+	+	+	±	−	−	−

Suspensions: 1—untreated.
2—heated 10 min. at 80°.
3—heated 10 min. at 80° and treated with pancreatic extract for 1·5 hr.

* The sera used were of homologous T type for the suspensions, namely, for **4**, a 'Wheatley' (*T*) serum; for **27** a 'T27' (*T*) serum; and for **46**, a 'T46' (*T*) serum.
Other conventions as in Table 1.

It seemed that, in some strains, heating to 100° decreased the T antigen content of the cells to an extent that they no longer yielded active extracts. It was then found that heating to 80° for 10 min. or to 60° for 30–60 min. was sufficient to increase the speed at which pancreatic extract caused loss of T agglutination without destroying the antigen (Table 5). Extracts from 18 hr. cultures grown at 37° in 500 ml. volumes of Todd & Hewitt broth, Pope & Smith broth, or nutrient broth with glucose, and heated in this way, gave specific precipitation with the homologous T antisera, when strains containing

T antigen and sera with a titre of T antibodies of 1/320 or more, as determined by slide agglutination, were used. The following Types were tested: **1**, **2**, **4**, **8**, **9**, **12**, **13**, **22**, **24**, **25**, **26**, **27**, **29**, **44**, and provisional Types **Richards**, **B3264**, **Impetigo 19**. For Types **24** and **26**, 1·5 l. volumes of culture were necessary and for Type **24** it was necessary to grow at 22° rather than at 37°. Elliott (1943) showed that growth at 22° enhanced the production of T antigen of Type **28**. Types **1**, **2**, **8**, **25**, **44**, and **Impetigo 19** yielded satisfactory extracts from 250 ml. cultures.

The amount of T antigen in the extract was not affected by heating for 30 min. at 56°, nor by 10 min. at 80°; it was greatly decreased after 10 min. at 100° at pH 7–8, and often almost completely inactivated.

Demonstration of cross-reactions due to T antigen by slide agglutination and precipitin tests

The cross-reactions due to shared T antigens among a set of strains of Types **4**, **24**, **26**, **28**, **29**, and **46**, were first demonstrated by Stewart *et al.* (1944) by agglutination, and by Lancefield & Dole (1946) by precipitation. I was able to confirm these results when the same strains were tested by the slide agglutination method (Table 6), except that no T-containing antisera were available for Types **28** and **29**. The same cross-reactions were observed by precipitin tests with extracts prepared by heat and treatment with pancreatic extract as described above.

In order to demonstrate the similarity of the extracts prepared by heat and pancreatic extract treatment to those studied by Lancefield & Dole, attempts were made to demonstrate parallel cross-reactions by slide agglutination and T precipitin tests with other groups of strains which are suspected, from experience of slide agglutination typing, to have common T antigens. The results from three such groups are shown in Tables 7–9. It is clear that in the **12/27/44** (Table 7) and **8/25/Imp.19** (Table 8) sets, the T extracts gave precipitates with those sera agglutinating the streptococcal suspensions to titres of around 1/160 or more, and in the **8/25/Imp.19** set, the cross-reactions, having titres lower than this, were not shown by the precipitin method. The results in the **Richards/13/B3264** (Table 9) set, although not so clear, were substantially the same.

DISCUSSION

It seems clear from the present work that the ease with which the T antigen can be destroyed by heat depends partly on the strain (as shown for example by the difference between the strains of Types 4 and 29; Table 1) and partly on the medium in which the cocci are grown. This point clearly needs much further investigation since it is of importance both in practice for obtaining the best absorbing suspensions for making single-antibody sera, and for the theoretical information that it may give about the surface structure of the streptococci. That the 'destruction' of the T antibody is more complex than at first appears is further shown by the additive effects of heat and treatment

Table 8. *Related T antigens in Types* 8, *Impetigo* 19, *and* 25 *shown by agglutination and precipitation tests*

	Sera																							
	8 'SF4' *T*								'Impetigo 19' *T*								25 'Matthews' *T*							
		Dilution								Dilution								Dilution						
		Agg.								Agg.								Agg.						
Strains	Ppt.	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Ppt.	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Ppt.	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
8, 'SF4' *T*	++	+++	+++	+++	+++	+++	+++	++	—	++	++	—	—	—	—	—	—	+++	++	++	—	—	—	—
'Impetigo 19' *T*	—	++	±	—	—	—	—	—	+++	+++	+++	+++	+++	+++	++	++	—	++	++	—	—	—	—	—
25, 'Matthews' *T*	—	++	++	++	—	—	—	—	—	+	—	—	—	—	—	—	++	+++	+++	+++	+++	+++	++	++

Ppt. = T precipitation test with undiluted serum; Agg. = slide agglutination test with serum at specified dilutions. Other conventions as in Table 1.

Table 9. *Related T antigens in Types Richards,* 13, *and B*3264 *shown by agglutination and precipitation tests*

	Sera																							
	Richards 'GIII' *M & T*								13 'Glover' *T*								'B3264' *T*							
		Dilution								Dilution								Dilution						
		Agg.								Agg.								Agg.						
Strains	Ppt.	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Ppt.	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Ppt.	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Richards 'GIII' *M & T*	++	+++	+++	+++	++	++	±	—	+	+++	++	+	+	—	—	—	—	+++	+++	+++	++	±	—	—
13, 'Glover' *T*	—	+++	++	++	+	—	—	—	+++	+++	+++	+++	+++	+	—	—	—	++	+	—	—	—	—	—
'B3264' *T*	—	+++	+++	+++	+	+	—	—	++	+++	+++	++	++	±	—	—	+++	+++	+++	+++	+++	+	+	+

Ppt. = T precipitation test with undiluted serum; Agg. = slide agglutination test with serum at specified dilutions. Other conventions as in Table 1.

Table 8. *Related T antigens in Types 8, Impetigo 19, and 25 shown by agglutination and precipitation tests*

	Sera																		
	8 'SF4' *T*								'Impetigo 19' *T*										
		Agg. Dilution								Agg. Dilution									
Strains	**Ppt.**	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	**Ppt.**	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	**Ppt.**	1/20	1/40
8, 'SF4' *T*	++	+++	+++	+++	+++	+++	+++	++	—	++	++	—	—	—	—	—	—	+++	++
'Impetigo 19' *T*	—	++	±	—	—	—	—	—	+++	+++	+++	+++	+++	+++	++	++	—	++	++
25, 'Matthews' *T*	—	++	++	++	—	—	—	—	—	+	—	—	—	—	—	—	++	+++	+++

Ppt. = T precipitation test with undiluted serum; Agg. = slide agglutination test with serum at specified dilutions. Other conventions as in Table 1.

Table 9. *Related T antigens in Types Richards, 13, and B3264 shown by agglutination and precipitation tests*

	Sera																		
	Richards 'GIII' *M & T*								13 'Glover' *T*										
		Agg. Dilution								Agg. Dilution									
Strains	**Ppt.**	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	**Ppt.**	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	**Ppt.**	1/20	1/40
Richards **'GIII'** ***M & T***	++	+++	+++	+++	++	++	±	—	+	+++	++	+	+	—	—	—	—	+++	+++
13, 'Glover' *T*	—	+++	++	++	+	—	—	—	+++	+++	+++	+++	+++	+	—	—	—	++	+
'B3264' *T*	—	+++	+++	+++	+	+	—	—	++	+++	+++	++	++	±	—	—	+++	+++	+++

Ppt. = T precipitation test with undiluted serum; Agg. = slide agglutination test with serum at specified dilutions. Other conventions as in Table 1.

with pancreatic extract. Furthermore it has been found that antisera prepared in this laboratory, by using as antigen autoclaved (one case) or boiled vaccines, can induce the formation of T antibodies in rabbits.

The preliminary heating of the streptococci before treatment with pancreatic extract appears to offer a useful method for increasing the yield of soluble T antigen, and with most of the limited number of strains tested the unconcentrated supernatant fluid from digestion (in 1·5–2·5 ml.) of the cocci from 500 ml. broth has given satisfactory precipitates with sera of adequate titre. Such extracts show cross-reactions between different sera of the same pattern as are found in slide agglutination tests and thus behave in the same way as the extracts obtained by Lancefield & Dole (1946).

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The Effect of Optically Active Forms of Some Amino-Acids on the Response of Yeast to β-Alanine and Pantothenic Acid

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SUMMARY: The growth-promoting action of β-alanine on *Saccharomyces carlsbergensis* and two strains of *S. cerevisiae* is stimulated or inhibited to different extents by the optical enantiomorphs of methionine, glutamic acid, asparagine and α-alanine. This behaviour can be considered as due, in each individual case, to one or more of three separate effects: (*a*) stimulatory, (*b*) inhibitory, and (*c*) antagonistic. In general, the L-forms of the amino-acids have a more pronounced action than the D-isomers. Racemic mixtures have intermediate effects. Similar but less striking results are observed when β-alanine is replaced by pantothenic acid. Atypical effects are obtained with D-alanine and D-glutamic acid.

Amino-acids exert an inhibitory effect on the growth of certain organisms in the presence of β-alanine, as shown by Sarett & Cheldelin (1945) and others. Hartelius (1946) found that L-glutamic acid and L-asparagine had a depressant action on the growth of yeast when the ratio α-amino-acid : β-alanine was higher than a critical value, below which a stimulatory effect was observed. In order to obtain more information concerning the mode of interference of α-amino-acids with the metabolism of β-alanine, the effects of both the D- and L-forms of a number of amino-acids have been studied and compared with the action of the corresponding DL-mixtures.

Previous workers have described several instances where D-amino-acids inhibited the growth of micro-organisms, while the L-isomers were inert. Thus, Davis & Maas (1949) found that inhibition by DL-serine of the growth of *Escherichia coli* was due to D-serine only. Similarly Mueller & Miller (1949) demonstrated that D-serine inhibited the formation of tetanus toxin. It was therefore expected that the action of the optical enantiomorphs on β-alanine would be unequal, and that, in general, the effect of the racemic form would be intermediate between the two. Results given in this paper support this idea.

METHODS

Growth Tests

Response curves were obtained by growing yeast in defined culture media containing graded amounts of β-alanine or calcium D-pantothenate and plotting dose against growth response. Control curves with no addition of α-amino-acid were compared with others after addition of fixed concentrations of D-, L- and DL-amino-acids.

Organisms and inocula. The test organisms were *Saccharomyces carlsbergensis* 4228, and two strains of *S. cerevisiae*. These were maintained in a liquid culture medium containing 16 % (w/v) malt extract, transfers being made to malt wort-agar slopes 24 hr. before the test was commenced. The inoculum was prepared by suspending a loop of yeast from a slope in saline, measuring the yeast content of this suspension by means of a Spekker absorptiometer, and diluting with saline to a concentration of 100 μg. moist yeast/ml.

Medium. The double-strength basal medium was essentially the same as that described by Atkin, Schultz, Williams & Frey (1943), except that pyridoxine hydrochloride replaced calcium pantothenate, and casein hydrolysate was omitted. It had the following composition: (g.) glucose, 40; potassium citrate, 20; citric acid, 4; $(NH_4)_2HPO_4$, 2; KH_2PO_4, 1·1; KCl, 0·85; $CaCl_2$, 0·25; $MgSO_4.7H_2O$, 0·25; (mg.) $MnSO_4.4H_2O$, 5; $FeCl_3$, 5; inositol, 50; nicotinic acid, 5; pyridoxine HCl, 5; aneurin, 0·5; (μg.) biotin, 16; water to 1 l., the pH being adjusted to 5·0 with HCl or NaOH. A further modification, introduced by Hopkins & Pennington (1947), was the addition of a quantity of nicotinic acid equal in weight to the pyridoxine hydrochloride.

Procedure. Double-strength medium (5 ml.) was distributed in 50 ml. conical flasks. Into each of one series of flasks, used as standards, β-alanine (or calcium pantothenate) solution (2 ml.) was pipetted, the series of flasks containing in a typical experiment 0·25, 1, 4, 16 and 64 μg. of β-alanine (or the molar equivalent of calcium pantothenate); 2 ml. of water were then added. Three other series of flasks contained, instead of water, 2 ml. of D-, L- or DL-amino-acid solution, usually 4 mg./ml. Duplicate flasks were used in all cases. To each of one set of flasks, serving as blanks, were added 5 ml. of medium and 4 ml. of water, so that a check could be made on the absence of β-alanine from the medium. All flasks were plugged with cotton wool and sterilized at 5 lb./sq. in. for 15 min. After cooling and adding 1 ml. of inoculum, the flasks were shaken at a frequency of 100 strokes/min. through a horizontal distance of 1 in. for 17 hr. in an incubator at 30°. The inoculum and growth period were so chosen that the highest growth obtained was less than half the maximum possible in the medium used.

Measurement of growth and plotting of results. After incubation 1 drop of 10 % sodium azide was added to check growth, followed by 20 ml. of cold water; after mixing, the light transmission of the resultant yeast suspension was measured with a Spekker absorptiometer using neutral grey filter H508, and the readings were converted to mg. dry weight of yeast by means of a calibration curve. The duplicate results did not generally differ by more than 3 %. The weight of yeast dry matter in milligrams was plotted against the β-alanine or pantothenic acid concentrations on double-logarithmic graph paper.

Note on the resolution of amino-acids

As the D-forms of the amino-acids were not commercially available the racemic mixtures were resolved as follows:

Methionine. The enzymic method of Brenner & Kocher (1949), based on the fact that pancreatin will hydrolyse the isopropyl ester of L-methionine, but not of D-methionine, was strictly followed.

Glutamic acid. Essentially Fischer's (1899) method of resolving benzoyl-DL-glutamic acid with strychnine was used.

Asparagine. DL-Asparagine was allowed to crystallize slowly from water at room temperature. Well-shaped crystals of the active enantiomorphs were formed and these were separated mechanically by observing the shape of the crystal faces.

α-Alanine. DL-Alanine was benzoylated as described by Levy & Palmer (1942), and the benzoyl derivative was resolved with strychnine and brucine (Pacsu & Mullen, 1940).

RESULTS AND DISCUSSION

The results obtained with *S. carlsbergensis* are presented graphically in Figs. 1, 2 and 3. Similar responses obtained with both strains of *S. cerevisiae* indicated that there was no significant difference between the behaviour of the three yeasts under the experimental conditions described. Preliminary experiments were carried out to determine the most suitable range of doses. The graphs given are those for the regions of most characteristic effects. Internal replication in the tests was good, and in all cases smooth curves were obtained.

It is clear from the experimental evidence that the extent to which α-amino-acids stimulate or inhibit the response of yeast to β-alanine depends upon their optical configuration. All four acids studied depressed to a marked degree such growth as is obtained in the complete absence of β-alanine or pantothenic acid (Fig. 1). The L-acids are more effective than the D-isomers, with the DL-forms intermediate. At concentrations of β-alanine or pantothenic acid of $0{\cdot}28 \times 10^{-6}$M, when the proportion of α-amino-acid to growth factor was highest, the effects were in general similar. Methionine (Fig. 2A and B) depressed growth at all concentrations of pantothenic acid or β-alanine tested, and the relative effect of the enantiomorphs of this amino-acid remained unchanged with increasing concentration of growth factor. In another experiment not recorded, the isomers of methionine had the same relative inhibitory effects when the concentration of β-alanine was increased to $5{\cdot}5 \times 10^{-3}$M. In the experiments with glutamic acid (Fig. 2C and D) and asparagine (Fig. 2E and F) a second effect was involved at higher

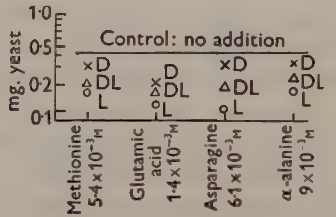

Fig. 1. Effect of D-, L- and DL-amino-acids on the response of *S. carlsbergensis* in the absence of pantothenic acid and β-alanine.

concentrations of β-alanine and pantothenic acid: in the presence of the L-amino-acids there was greater growth than in the standards.

D-Alanine (Fig. 2G and H) gave growth which was almost equal to the standard, suggesting that there may be some stimulatory action. This was confirmed by the fact that in one of the strains of *S. cerevisiae* there was a definite increase of growth above the standard. L-Alanine had a growth-depressant action even at relatively high concentrations of β-alanine, but at the highest levels this effect was largely overcome. The effect produced by D-alanine was similar, but only a sixfold increase of β-alanine concentration was required to produce the same growth as the standard, whereas the corresponding ratio for the L-isomer was sixty.

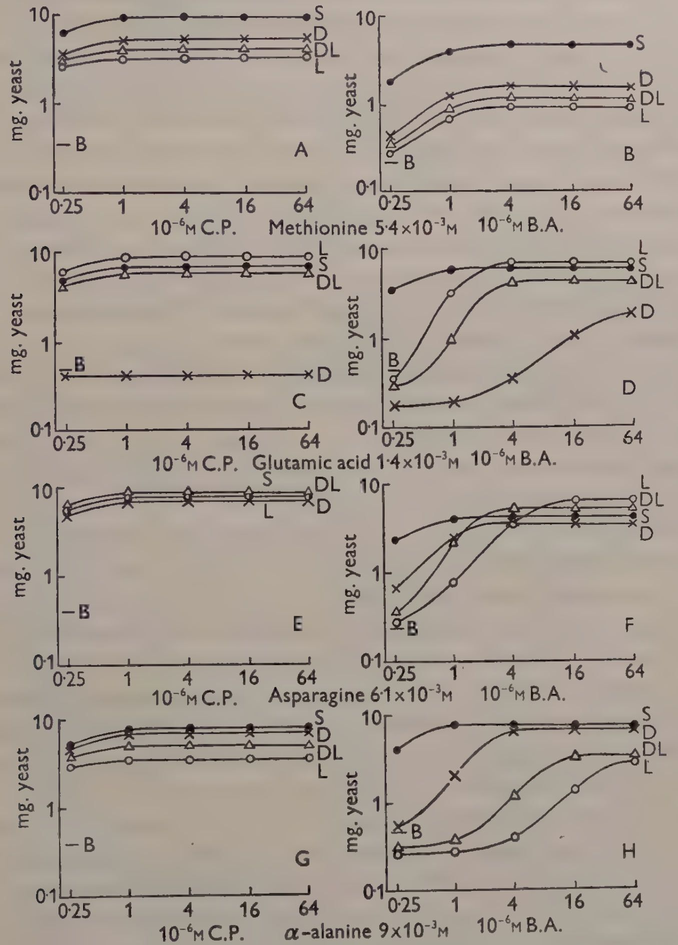

Fig. 2A–H. Effect of D-, L- and DL-amino-acids on the response of *S. carlsbergensis* to calcium pantothenate and β-alanine. C.P., calcium D-pantothenate; B.A., β-alanine; S, standard with no added α-amino-acid; B, blank for standard curve with no added calcium pantothenate or β-alanine.

In view of these facts, it seems necessary to distinguish between three distinct effects of the α-amino-acids.

(1) *Inhibitory effect.* The first and simplest case is illustrated in Fig. 2A and B, where the addition of $5 \cdot 4 \times 10^{-3}$M methionine depressed growth in the presence of both pantothenic acid and β-alanine to approximately the same extent throughout the range of concentrations. Graphically this is expressed by the fact that the log : log curves obtained on addition of methionine were below, and roughly parallel to, the standard curves. This would indicate that both the D- and L-forms of methionine had a depressant effect on the yeast growth, which was probably independent of the metabolic processes connected with β-alanine or pantothenic acid, the L-isomer being more active than the D-form. The effect can be considered as due to an unspecified toxic action on the yeast cells which may be connected with competitive inhibition of amino-acid or protein synthesis.

(2) *Stimulatory effect.* The second effect is shown in Fig. 2C, where L-glutamic acid stimulated growth above the standard. Probably the stimulatory effect was also independent of processes connected with the utilization of pantothenic acid or its synthesis from β-alanine, because it was constant at all higher doses of both growth factors, i.e. in complete medium. The effect is possibly concerned with nitrogen assimilation or protein synthesis (Thorne, 1949; White & Munns, 1950).

The stimulatory effect of the natural or L-isomers is not surprising, but the observation that D-alanine stimulated yeast growth is more difficult to explain. Snell and co-workers (Holden, Furman & Snell, 1949; Holden & Snell, 1949) observed that D-alanine could replace vitamin B_6 for the growth of certain lactic acid bacteria. Growth of yeast in pyridoxine-free medium, with and without D-alanine, did not reveal any connexion between the two factors; in fact, using one strain of *S. cerevisiae*, D-alanine was stimulatory to the same extent at all levels of pyridoxine, pyridoxal or pyridoxamine in the presence of an optimal concentration of pantothenic acid, which indicates that the effect was independent of vitamin B_6. It can only be suggested that D-alanine is required for some reaction within the cell and cannot be synthesized sufficiently rapidly to allow the yeast to grow at its maximum rate.

(3) *Antagonistic effect.* The third condition is illustrated in Fig. 2H, which shows the effect of α-alanine in the presence of β-alanine. This action of α-alanine (described above) is represented graphically by typically hysteresis-shaped curves. This type of response is characteristic of the action of a metabolic antagonist, and is taken to express the fact that an enzyme concerned in the chemical processes connected with the formation or function of the growth factor is blocked by a compound of similar molecular structure when present in large excess, but as the ratio of antagonist to growth factor decreases (i.e. at high concentrations of growth factor) the inhibitory effect is overcome and growth approaches normal. In this case, the enzyme concerned can most easily be visualized as that which catalyses the reaction by which β-alanine combines with pantoic acid to form pantothenic acid, because the antagonistic effect was never observed in the presence of pantothenic acid

alone. α-Alanine, being chemically similar to β-alanine, might be expected to have a strong antagonistic action, and it is striking that in these experiments, the greatest antagonistic effect was observed with this amino-acid. Further, in view of many similar observations by workers in this field, the natural L-isomer would be most likely to have the more pronounced effect. As noted above, the inhibition ratio of L-alanine to β-alanine was only about a tenth of that of D-alanine to β-alanine. Similar, but smaller, effects were shown by L-glutamic acid (Fig. 2D) and by L-asparagine (Fig. 2F) in the presence of β-alanine. In these cases only about four times the usual concentration of β-alanine for optimal growth was required to overcome the antagonistic effect. If it be accepted that the strong antagonistic action of L-alanine to the growth of yeast, in the presence of β-alanine, but not pantothenate, is due to the structural similarity of the α- and β-forms, an explanation of the antagonistic effects of L-glutamic acid and L-asparagine may be sought in the conversion of these amino-acids to α-alanine by the reactions in which asparagine is converted to aspartic acid by asparaginase, and aspartic and glutamic acids to α-alanine by transaminases. These enzymes are specific for the L-isomers of the amino-acids.

Combinations of the inhibitory, stimulatory and antagonistic effects described above would explain all the responses obtained, except that of D-glutamic acid, which is discussed below. The effects on the response to pantothenic acid of D- and L-methionine (Fig. 2A), and L-alanine (Fig. 2G), are examples of inhibition only. D- and L-Methionine (Fig. 2B), and D-asparagine (Fig. 2F) had a similar action in the presence of β-alanine. D- and L-Alanine had a clear-cut antagonistic effect in the presence of β-alanine (Fig. 2H), the action of the L-isomer being stronger than that of the D-form. This action was combined with a small inhibitory effect throughout the entire range, and in the case of the D-isomer, a slight stimulatory effect was superimposed at high concentrations of β-alanine. L-Glutamic acid (Fig. 2C) had a stimulatory action on the response to pantothenic acid, except at very low levels of pantothenic acid (Fig. 1). L-Glutamic acid (Fig. 2D) and L-asparagine (Fig. 2F) both had a stimulatory effect on the response to β-alanine, upon which was superimposed an antagonistic action.

The case of D-glutamic acid was extraordinary in that growth was strongly inhibited throughout the full range of pantothenic acid used (Fig. 2C), whereas L-glutamic acid stimulated, and the DL-mixture had an action very similar to that of the L-isomer. On the other hand, in the presence of β-alanine (Fig. 2D), the inhibitory effect of the D-form was decreased at high levels of β-alanine. This observation was confirmed by carrying out a special experiment in order to exclude all variables such as different inocula, and slight differences in temperature or time of incubation. Fig. 3 illustrates the effect of varying the concentration of D-glutamic acid on the response to β-alanine. It will be seen that with increasing amounts of glutamic acid the effect is qualitatively similar, but greater in degree. At first sight this behaviour was unexpected, because if β-alanine be converted to pantothenic acid before it can exert a growth-factor effect, it would be expected that the response to β-alanine would be depressed to as great an extent as the response to pantothenic acid. However, the following explanation of the mechanism is presented in view of further experimental evidence.

It has been shown that pantothenic acid can form a conjugate with glutamic acid, presumably in the L-form (King & Cheldelin, 1948; King, Fels & Cheldelin, 1949), and it is possible that such a compound must first be formed before pantothenic acid can function as a growth factor. In such a case, D-glutamic acid might be expected to be an antagonist to the enzyme system which effects the conjugation. Evidence for this was obtained by using mixtures of the L- and D-forms in varying proportions in tests with pantothenic acid. It was found that a small proportionate addition of the L-isomer would neutralize the depressant effect of the D-form, suggesting a competitive inhibition of the action of the enzyme, which has a much stronger affinity (of the order of fiftyfold) for the L-acid than for the D-isomer. Further investigation showed that three different D-amino-acids (methionine, asparagine and α-alanine) added in the ratio of 1 part amino-acid to 9 parts of D-glutamic acid (total weight 2 mg./flask) had no significant effect in removing the inhibition, whereas similar additions of the L-forms, and of glycine and β-alanine which do not contain an asymmetric carbon atom, removed the inhibition almost as effectively as L-glutamic acid. Thus it is concluded that at lower levels β-alanine functions as a growth factor, being converted into pantothenic acid, but that at high levels it performs a second function in relieving the inhibitory effect of D-glutamic acid, and therefore produces the characteristically-shaped curve of Fig. 2D, as compared with the total inhibition in the presence of pantothenic acid only (Fig. 2C).

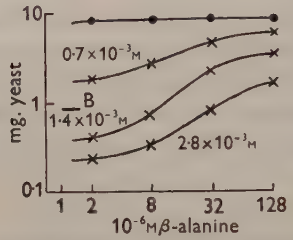

Fig. 3. Effect of varying concentrations of D-glutamic acid on the response of *S. carlsbergensis* to β-alanine; B, blank for standard curve with no added calcium pantothenate or β-alanine.

Various workers have suggested mechanisms for the inhibitory effect of α-amino-acids on β-alanine metabolism. Nielsen, Hartelius & Johansen (1944) considered that the following two reactions were competing:

$$\beta\text{-alanine} + \text{pantoic acid} \leftrightharpoons \text{pantothenic acid},$$
$$\text{glutamic acid} + \text{pantoic acid} \leftrightharpoons \text{pantoylglutamic acid}.$$

Glutamic acid would, therefore, tend to decrease the amount of pantothenic acid that could be formed. Later Hartelius (1946) proposed a further side reaction in which a dipeptide was formed from β-alanine and the interfering amino-acid:

$$\beta\text{-alanine} + \text{glutamic acid} \leftrightharpoons \text{dipeptide}.$$

The dipeptide was assumed to be inactive, i.e. it could not form an active pantoyl compound. Neither of these processes would account for inhibition when pantothenic acid replaces β-alanine. It would seem better, therefore, to postulate that inhibition is caused primarily through competitive interference by the inhibitor with certain specific side chains of the active enzyme molecules, with a consequent displacement of the substrate. Such inhibition is discussed by Rydon (1948) in greater detail.

A diversity of opinion exists on the subject of stimulation. Ehrlich's and Stickland's work in this field is well known and has been summarized by Thorne (1949). It has been suggested by Thorne, and also by White & Munns (1950) that in certain circumstances amino-acids are assimilated intact by growing yeast. This explanation would only cover the stimulatory effect of single amino-acids if it were assumed that the majority of the amino-acids could be synthesized sufficiently rapidly to have no restricting effect on yeast growth, whilst a small number, such as glutamic acid and asparagine, could not be formed at this rate. This can only be partially true at best, because the addition of all the amino-acids known to be required by yeast results in a faster rate of growth than is produced by the few which are stimulatory alone. At present the experimental evidence is insufficient to enable exact conclusions on the mechanism of inhibition or stimulation to be drawn.

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Adenosine and Spore Germination; Phase-contrast Studies

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SUMMARY: Phase-contrast microscopy enables germination to be studied in the spores of *Bacillus cereus* and *B. subtilis* a few minutes after the addition of adenosine; by this means adenosine can be identified in minute concentrations. Adenosine in high concentrations is, however, inhibitory to vegetative growth.

Adenosine appears to act as an indicator of conditions suitable for vegetative existence, and induces, almost instantaneously, profound physical changes in spores. The analogy with hatching of eel-worm and amoebic cysts, also susceptible to specific excitants, is stressed.

In recent papers Hills (1949*a* and *b*, 1950) reviewed the literature relating to spore germination and showed the primary importance of adenosine. He incubated spores in a chemically-defined medium, heated them for 15 min. at 60°, and counted the spores remaining viable. When the viable count fell it was assumed that some spores had germinated in the test fluid. The results were submitted to statistical analysis, and indicated that adenosine was of primary importance for initiating germination. This interesting observation has been confirmed by the experiments here described, using a different technique, namely, examination of the germinating spores directly by phase-contrast microscopy.

TECHNIQUE

Spore suspensions of *Bacillus cereus* and *B. subtilis* isolated from a compost heap were prepared by surface growth at 37° on the medium of Knaysi & Baker (1947) for electron microscopy, containing glucose 0·2 g. and an equi-molar mixture of KH_2PO_4 and K_2HPO_4, 0·23 g. in 100 ml. of distilled water. This was modified by the addition of 0·5 g. NaCl and 1% New Zealand agar (Davis Gelatine Ltd., London). On this medium growth was profuse and spore formation excellent, and for practical purposes in a few days no vegetative forms remained. The spores were washed in saline, centrifuged and resuspended in small volumes of saline. After heating at 80° for 15 min. they were kept in a refrigerator until used. They did not germinate in the saline, and remained capable of germination in suitable media for at least three months.

Slide cultures of the organisms were prepared by making streaks from 24-hr. cultures on nutrient agar on to a cover slip. After drying in air for 15 min. the melted agar medium of Knaysi & Baker at 50° was dropped on to the cover slip, which was mounted on a slide, and sealed with wax. Within a week in most cases very large numbers of spores had formed; those slides in which spore formation was poor were discarded (Pl. 1, fig. 1).

The germination of spores was observed by phase-contrast microscopy. The method was developed for making cinema films of bacterial growth phases, for which it was essential that growth should be in two planes only and that Brownian movement should be eliminated. After spores had been formed in a slide culture the cover-slip was removed with a scalpel; the agar adhered completely to the cover-slip. One half of the agar was cut away, and the cover-slip replaced and sealed on three sides. Hartley's broth was run in on the fourth side, where the agar had been removed, and this side also was then sealed. Germination was observed under a 1/12 in. objective by phase contrast, the microscope being mounted in an incubator at 37°.

Within 15 min. the spores, which originally appear as translucent oval bodies with a blue sheen and very thin walls quite suddenly become completely opaque, and appear, as do all young bacteria by phase contrast, homogeneously black, or very dark blue (Pl. 1, fig. 2). In the case of *B. cereus* the opacity develops symmetrically and very suddenly, as an over-exposed photographic plate darkens in a developer. There is no sign of a focal point from which a dark area expands. After this darkening changes are slow for 1 hr. but the spore enlarges in all dimensions. The ghost of the bacillus from which the spore develops often becomes visible, and into this shroud the new bacillus grows. Within 2 hr. a stumpy bacillus has formed; no capsule bursts, and no husk is shed. Division then takes place; subsequent phenomena will not be considered here.

In the case of *B. subtilis* the initial darkening appears to expand from a lateral focus. The spore then becomes bean shaped; the convex side appears to be that on which the lateral focus was first visible, but since the beans may lie in any position it is difficult to be sure of this. A small spherical protrusion appears in the centre of the convexity, and rapidly assumes bacillary form. The bacillus grows out at right angles to the spore axis, and soon the spore becomes again transparent, except where the bacillus intrudes into its cavity. Division takes place after 2 hr. and soon empty bean-shaped husks with lateral pores may be seen.

Many other spores of species belonging to the *Bacillus* group have been studied, and those of *Clostridium sporogenes*. In these cases no attempt was made to study the effect of adenosine, but in every case darkening of spores took place in suitable media very quickly.

Certain facts should be emphasized. Spores which do not darken never develop into bacteria. When the fluid diffused into the agar is a fully nutrient medium, such as nutrient meat broth, practically all spores darken, and of those that darken, practically all form bacilli which divide. In any preparation less than 1 % of the spores are initially dark. Other studies not detailed here indicate that these are imperfectly-developed spores; they do not form bacilli. It is therefore safe to say of translucent spores that darkening is the first and the essential stage in germination, and may in itself be accepted as proof that the state of dormancy is ended.

It is difficult to see any change in unstained preparations without phase contrast, but spores which have darkened can be stained with methylene blue or other simple bacteriological stains; this observation confirms those of Howie & Cruickshank (1940). A warning should be given that in old slide cultures bacterial debris is often visible. Many substances in concentrations as low as 10 mg./ml. can initiate normal growth in these distorted remnants; but these substances do not darken spores. If, however, the preparations are not continuously watched, it is easy to conclude falsely that spores have germinated, as the cultures teem with bacteria in a few hours. Examples of such substances are ammonium salts and vitamin B_{12}, neither of which can initiate spore germination, but both of which revitalize 'spent' cultures.

The technique described is admirable for photography, but a less elaborate and more rapid technique gives equally clear cut results. A platinum loopful of the heated spore suspension is placed on a slide and mixed with a loopful of a test reagent. When sealed under a cover-slip spore darkening and bacterial development can be studied by phase contrast, and negative results recorded after 2 hr. In this way a very large number of tests can be quickly made. Most of the experiments described here were performed with this technique and confirmed by the slide-culture test when positive.

EXPERIMENTS

Since it appeared that spore darkening was an essential stage, and the first visually determinable in spore germination, experiments were performed to discover what substances caused it. It soon became obvious that media in which vegetative forms grew readily for many sub-cultures did not necessarily induce spore germination. For example, the simple medium used by Stokes & Woodward (1943) containing glucose, asparagine and inorganic salts supports growth of *B. cereus* and of *B. subtilis*, but spores do not darken in this medium, and the medium inoculated with spores develops no opacity. These spores will not form visible growth on Knaysi & Baker agar, a medium used for making cinematographic records of the growth phases of the vegetative forms up to and including spore formation. Nor will spores darken with Bacto-Vitamin Free Casamino Acids (Baird & Tatlock Ltd., London), in which excellent growth of the vegetative forms develops.

Spores quickly darken however in media such as Hartley's or Lemco broth. The speed of darkening and percentage of spores darkening are proportional to the concentration of the broth; in this way the concentration of the active substance may be roughly estimated over a standard time of 2 hr. Thus with undiluted Hartley's broth all spores are dark within 15 min.: at a dilution of 1/10 not more than 5 % are dark in 2 hr. and none at a 1/100 dilution in saline.

Spores also darken in broth containing antibiotics at concentrations which cause wide inhibition zones of vegetative bacilli when diffused from cylinders into pour plates. Streptomycin at 100 units/ml. and aureomycin or chloromycetin (chloramphenicol) at 0·1 % produce zones of 20 mm. diameter or more with shake cultures with this technique; but when made up in Hartley's broth they do not inhibit spore darkening. At these concentrations however spores never develop into bacilli; at 1/10 of these concentrations bacilli develop, but multiplication ceases when two or three bacilli have formed from one spore. That is, spores will darken in media where vegetative growth is impossible; they will not darken in other media in which vegetative growth readily occurs. The chemical basis of spore germination differs therefore from that of vegetative growth.

Since the *B. cereus* and *B. subtilis* strains used were isolated from decaying vegetables the spore-darkening factor was first sought in vegetable extracts obtained by boiling; it was found in cabbage, lettuce and potatoes. It was next found in newly-purchased Marmite and Yeastrel, and in boiled extracts of compressed yeast tablets; there was only a trace in a five-year-old sample of Yeastrel.

The active principle could be dialysed through collodion sacs, and could be purified and concentrated by precipitating aqueous solutions of Marmite and Yeastrel with ethanol, the factor remaining in solution. The ethanolic solution was evaporated, the residue taken up in distilled water, and the solution precipitated in acetone; the factor remained in solution. When the acetone solution was passed through a column of aluminium oxide the factor was adsorbed, and much inactive material was not. The top section of the column was eluted with 90% (v/v) ethanol in water. This ethanol solution was again evaporated, and the yellow deposit taken up in distilled water. The pH value of the solution was 2; and at this pH was inactive; but when adjusted to pH 7·2 it was very active, since spore darkening was almost instantaneous. The biuret reaction was negative. These experiments eliminated many possible compounds; many other substances were tested. The factor was absent from Bacto-Vitamin Free Casamino Acids and from gelatin peptone: some proprietary peptones such as that of Evans, Lescher, Sons & Webb contained it; Gurr's peptone did not. It was present in human serum and absent from a sample of human urine. Fourteen amino-acids were tested in an arbitrary mixture and DL-alanine, L-tyrosine, DL-cysteine, DL-tryptophan and methionine separately, all with negative results.

Negative results were also obtained with aneurin, riboflavin, nicotinic acid, pantothenic acid, folic acid ('Folvite', Lederle), choline and biotin (tested separately and together), ascorbic acid, several samples of vitamin B_{12} and of therapeutic liver extracts, adenine, guanine, creatine, creatinine and uric acid.

Thus meat extracts and digests, vegetable extracts and yeast extracts contained the factor; from yeast (and later cabbage) ethanol- and acetone-soluble, heat-stable and dialysable materials which cause spore darkening have been obtained, but no single substance or group of substances had proved to be positive.

Our negative results confirmed Hills' findings, and when adenosine was tested we found at once that it caused spore darkening. A sample from British Drug Houses was very active at 0·1% in distilled water against washed spores suspended in saline; it was usually inactive at 0·01% and always inactive at lower concentrations. When tested by diffusing it into spores formed in slide culture on Knaysi & Baker agar it caused spore darkening at a concentration of 16 μM in M/30 phosphate at pH 7·3. The spores not only darkened, but bacilli also formed, and divided once or twice; they then lysed rapidly and in 24 hr. with *B. subtilis* only empty spore-husks remained (Pl. 1, figs. 3–6); both *B. cereus* and *B. subtilis* spores germinated in this way.

Since the newly-formed bacilli lyse within 24 hr. it is not surprising that visible growth zones cannot be obtained on Knaysi & Baker agar plates, pre-seeded with suspensions of spores, when adenosine or the ethanol and acetone soluble fractions of yeast are diffused. This method, analogous to that used for many biological tests for vitamins is at first sight attractive, and excellent growth rings are obtained by diffusing complete media such as Hartley's broth. Since the vegetative form will grow on Knaysi & Baker agar, it appears that newly-germinated bacilli are more exacting in their growth requirements than bacilli developing from vegetative forms. However, in any sub-culture of vegetative forms in slide culture, studies by phase contrast indicate that only a minority of bacilli do in fact reproduce; the remainder lyse and in so doing may set free growth factors.

The effect of adenosine is enhanced by Bacto-Casamino acids (vitamin free). In this way adenosine at a dilution of 1/200,000 could be detected but not at 1/2,000,000. Since equal volumes (1 platinum loopful) of spore suspensions and of test fluid were used the dilution at which adenosine can be detected is at least 1/200,000; the volume in which this concentration is present is determined mainly by the size of the platinum loop used.

Table 1. *Influence of Bacto-Casamino acids on spore-darkening effect of adenosine*

B. cereus spores, washed and suspended in 0·85 % saline. Heated for 10 min. at 80°. Duration of experiment 2 hr.

A. Adenosine in 1 % Casamino Acids, M/30 phosphate buffer, pH 7·2:

Adenosine (%)	
0·1	++
0·01	++
0·001	++
0·0001	Negative
0·00001	Negative

B. Adenosine in M/30 buffer, pH 7·2:

Adenosine (%)	
0·1	++
0·01	Negative
0·001	Negative
0·0001	Negative
0·00001	Negative

Controls

Saline	Negative
Hartley's broth	++
M/30 phosphate buffer	Negative
1 % Casamino Acids and buffer	Negative

Hills (1949*a*) suggested that microbiological assay of adenosine might be possible by measuring its effect on spore germination. By his technique it gave a significant effect at 0·2 μg./ml.; this contrasts with other micromethods quoted by him which estimate 0·1 μg. The amount of adenosine which can be detected by the spore-darkening technique depends on the smallness of the drop examined. In the experiment shown in Table 1, the amount of adenosine which gave a strong positive result was 0·025 μg. and completely negative results were obtained with 0·0025 μg. Since the experiments only occupy a few minutes, and results are read in 2 hr., the method has obvious advantages. However, although no other substance was found which produced the same effect as adenosine, it is too early to say that its properties are unique.

It is surprising, in view of the importance of adenosine in spore germination, to find that it is inhibitory to growth of vegetative organisms in concentrations higher than 0·006 %. During the course of experiments with Casamino Acids+adenosine mixtures it was noted, on one occasion, that slide preparations containing high concentrations of adenosine (over 0·001 %) showed darkened spores, but no bacilli after 4 hr., but that below this concentration, while the spores were still translucent, there were many actively-multiplying bacilli. It was found that the Casamino Acids solution had become contaminated, although no visible haze was present. Therefore it appeared that the very small inoculum contaminating this solution had not grown in the higher concentration of adenosine, but had grown in the lower. This was shown to be the case by making doubling dilutions of 0·1 % adenosine in buffered Casamino Acids solution, and inoculating the fluid with a suspension of a 24-hr. agar culture of *B. cereus* washed in saline. There was very poor growth in the first four tubes but dense growth and a rich deposit in lower dilutions, and in the control containing buffered Casamino Acids solution only. When a nutrient medium such as Hartley's broth is diffused into pour plates of *B. cereus* in Knaysi & Baker medium a dense growth zone is found. But in many cases the zone begins 4 mm. or more from the cylinder. This central inhibition may perhaps be due to an inhibitory concentration of adenosine.

DISCUSSION

The dormant state is widely found in living creatures though difficult to define. It varies from the natural sleep of animals and the quiescence of the wintering tree to the cysts of worms and amoebae and the spores of bacilli. To each dormant state there is an appropriate excitant, announcing, like the kiss in the fairy tale of the Sleeping Beauty, that the environment is now suitable for greater and different activity, including reproduction. Thus Calam, Raistrick & Todd (1949) in the case of eel-worm cysts, and Crump (1950) with the amoebic cyst, have found indications that a specific excitant will alone awake the inert organism to vegetative existence.

The bacterial spore is admirably adapted to prolonged periods of rest, resistant as it is to heat, desiccation and adverse chemical environments. But

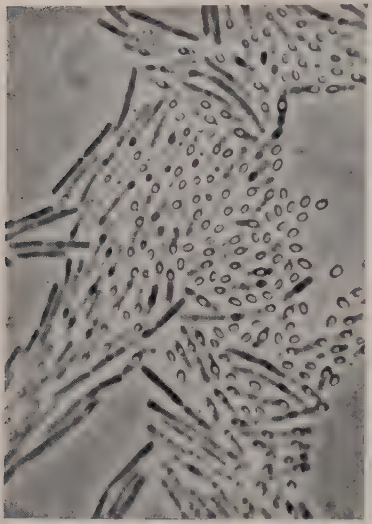

Fig. 1

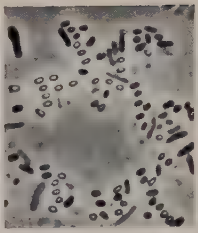

Fig. 3

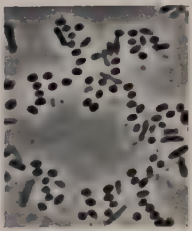

Fig. 4

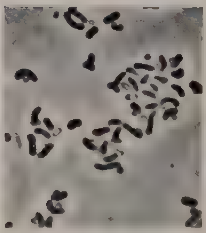

Fig. 5

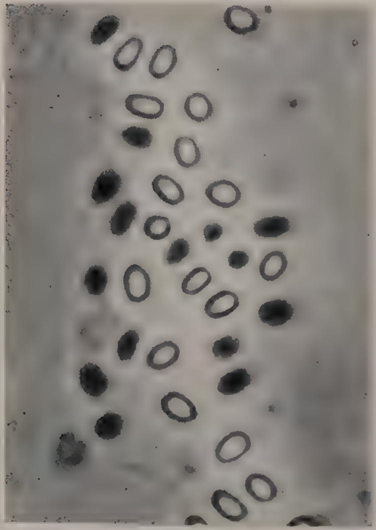

Fig. 2

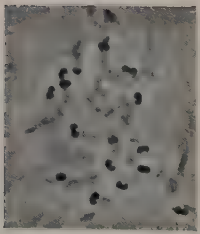

Fig. 6

R. J. V. PULVERTAFT & J. A. HAYNES—ADENOSINE AND SPORE GERMINATION; PHASE-CONTRAST STUDIES. PLATE 1 these protective mechanisms would be of little advantage were it not also provided with a chemical criterion of the environment adequate for vegetative life and reproduction; were it otherwise it might leave the security of its dormant state to starve in a desert. Adenosine is the only known substance accepted by the spores studied as this criterion. The change that occurs when it is present converts it from an object in the border-land between the living organism and lifeless organic material to a recognizably living and reproducing cell; it changes its refractive index and its permeability almost immediately. It would be profitable to analyse chemically the change which occurs under these circumstances.

Our thanks are due to Dr H. P. Chu of Cambridge for help in the identification of the strain of *B. cereus* used.

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EXPLANATION OF PLATE

Fig. 1. Slide culture, *B. cereus*. Early spore formation. ×1,200.

Fig. 2. Slide culture, *B. cereus*. Spores 5 min. after diffusion of Hartley's broth, showing dark spores. ×3,600.

Fig. 3. Slide culture, *B. subtilis*. Before diffusion of 0·1% adenosine in distilled water. ×1,200.

Fig. 4. The same field 15 min. after diffusion of adenosine. Spore darkening. ×1,200.

Fig. 5. Another field from the same preparation, showing lateral emergence of bacilli, 4 hr. later. ×1,200.

Fig. 6. The same field 24 hr. later, showing spore husks with lateral pore and lysis of bacteria. The focus is adjusted to define the pore; in sharp focus the husk is translucent.

All slide cultures on Knaysi & Baker's agar.

Photomicrographs by Cooke, Troughton, and Simms phase-contrast microscope, ×95 objective, ×9 Zeiss ocular.

FIG. 2. Subsequently enlarged.

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The Action of the Ciliates of the Sheep's Rumen upon Various Water-soluble Carbohydrates, Including Polysaccharides

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SUMMARY: When holotrich ciliates of hay-fed sheep's rumen acted upon water-soluble carbohydrates under conditions comparable with those in the rumen save for the absence of appreciable bacterial competition, only glucose, fructose, sucrose, inulin, bacterial levan (from *Bacillus megatherium*) and to a lesser extent cellobiose were utilized for rapid and extensive storage of iodophilic polysaccharide granules (cf. Oxford, 1951). No other soluble carbohydrate tried (including maltose) was so utilized. When polysaccharide storage did take place the product always gave a purple colour with iodine, and had the properties of a starch rather than glycogen in that it gave an insoluble iodine complex under the conditions of Pucher, Leavenworth & Vickery (1948). Storage of starch by oligotrich ciliates was much slower than with holotrichs, and did not take place with cellobiose as substrate. Holotrich ciliates continued to replace themselves in the sheep's rumen even when large volumes of rumen contents were periodically withdrawn over a long period. The protozoan starch could be isolated in about 1% yield by application of the Pucher *et al.* (1948) method direct to the dry matter of strained rumen liquor taken from sheep which had grazed on starch-free Spring grass.

The ciliate literature contains numerous references to the storage of polysaccharides variously termed glycogen, starch, paraglycogen, paramylum, etc. (cf. Doyle, 1943; Kudo, 1947), but these iodine-staining inclusions have never been isolated in sufficient quantity to allow proper chemical investigation of them. The observation made previously (Oxford, 1951) that the holotrich ciliates of sheep fed on a starch-free diet (hay) stored relatively enormous numbers of tiny iodophilic polysaccharide granules when glucose, for example, was fermented *in vitro* by muslin-strained rumen liquor, has now been utilized in the preparation of a large specimen (more than 50 g.) of this polysaccharide in a relatively pure state in order that such a chemical investigation might be made, beginning with a decision between the starch and glycogen possibilities.

In these *in vitro* fermentations the protozoa were, of course, competing with bacteria for the available sugar, and the yield of protozoan starch was never more than 5–10% of the total sugar fermented. Attempts were, therefore, made to use an inoculum consisting mainly of protozoa, not only to minimize bacterial interference in order to obtain larger overall yields of protozoan starch, but also to find out which carbohydrates other than glucose, fructose and sucrose can be converted into starch by the protozoa acting alone. Owing to the difficulties of large-scale isolation of protozoa from rumen liquor without impairment of their fermentative powers, the first objective (greater overall yield) has not been attained, but the second, which obviously does not depend on the isolation of the greater part of the protozoa in rumen liquor, has largely been achieved and forms the main subject of this paper.

The experience gained was further applied to the direct isolation of protozoan starch from the rumen contents of grass-fed sheep. This is of some importance because it is well known that lush Spring grass, which is relatively rich in water-soluble carbohydrates and protein but almost free from starch, supports the greatest density of protozoan population in the rumen.

METHODS

Animals. Rumen-fistulated sheep Nos. 272 and 337 were used as previously described (Oxford, 1951). They were fed exclusively on meadow hay. In addition three unfistulated Cheviot ewes, each 1 year old, were put to grass from May to August and killed when required.

Fermentation procedures. In the large-scale preparation of protozoan starch the methods described by Oxford (1951) were modified as follows: (*a*) The fermentation time was decreased to 3 hr. This was sufficient for maximum starch storage in holotrichs but not in oligotrichs, for which an overnight fermentation is better. The separation of heavy holotrichs unmixed with oligotrichs was therefore facilitated. (*b*) The initial glucose concentration was decreased to 0·5 %. (*c*) Soft tap-water was used instead of distilled to make up the basal medium. (*d*) The inoculum was increased to 25 % (v/v) of total fermentation liquid. (*e*) All liquids were maintained at 40° throughout, including rumen contents while being strained. When this straining was done at room temperature the subsequent protozoan starch storage was much slower. (*f*) The working-up procedure was modified to enable an appreciable part of the starch-filled protozoa to be quickly isolated. Fermentations were carried out in 2-litre Buchner flasks, four at a time, and 30 min. after the last shaking the supernatant was carefully decanted and the residue, consisting mainly of the various protozoan bottom layers, was transferred to a number of 50 ml. separating funnels with short stems. After standing for 20 min. the bottom white layers in each were drawn off into boiling-tubes, and the heavy protozoa therein washed with distilled water, by decantation. The subsequent bursting of the cells with Teepol XL and the collection of starch granules was carried out as previously described (Oxford, 1951) save that a temperature of 50° was used. Not all the holotrich starch was isolated by this rapid procedure, but by rigid standardization of conditions it was hoped that yields in successive fermentations could be fairly compared.

Carbohydrate fermentations without appreciable bacterial competition. Owing to the slowness of their deposition and the difficulty of washing them free from bacteria it was not found practicable to carry out fermentations with washed total protozoa from muslin-strained rumen liquor (2 l.) but only with a small fraction of relatively bacteria-free protozoa. Quite a fair proportion of the total ciliates of the sample of rumen contents strained were, however, left on the muslin together with plant residues, and these protozoa were resuspended in the basal salt medium (Elsden, 1946) with acetate at 40° and separated from plant debris by re-straining. When this filtrate was allowed to stand the protozoa sank to the bottom quite rapidly, since the basal medium is less dense and viscous than rumen liquor. They could then be quickly collected, relatively free from bacteria, by decanting the supernatant. The usual procedure was to wash the residue on the muslin twice, combine the two protozoan layers so obtained, and make up to 150 ml. with basal medium. After thorough mixing, 75 ml. of this suspension were added to each of two 250 ml. Buchner flasks containing 700 mg. of glucose (or fructose) and the carbohydrate to be tested, respectively, dissolved in a little basal medium. After filling each flask with the basal medium the subsequent fermentation procedure was exactly as described above. Provided the fermentation was not allowed to proceed beyond 4–5 hr. no overwhelming turbidity due to bacterial growth developed. The same procedure was also used for the washed protozoa obtained direct from strained rumen liquor. When a polysaccharide was to be fermented it was first dissolved in a little warm water before addition of the inoculum.

Isolation of protozoan starch from rumen contents of normally grazing sheep. Glucose fermentations carried out with strained rumen contents from sheep slaughtered after grazing for some weeks on Spring grass led to no useful results, at least as far as protozoa were concerned. The fermentations proceeded with uncontrollable violence and owing to the physical properties of the rumen fluid did not result in the separation of a white protozoan bottom layer. Microscopical examination of the strained rumen liquor having shown that there was a rich fauna of iodophilic ciliates, the method of Pucher, Leavenworth & Vickery (1948), involving perchloric acid extraction of the starch, was directly applied to the residue remaining after steam-bath evaporation of strained liquor obtained by working up rumen contents within a few hours of slaughter, followed by drying *in vacuo*. Instead of 250 mg. of residue material as recommended, ten times this amount was used in each determination, in order to make possible the working up of the entire rumen contents. The perchloric acid method was also applied to various protozoan fractions obtained from hay-fed sheep; in these instances the method of Pucher *et al.* was not modified.

Substrates. The following substances were used: fructose (glucose free), galactose, mannose, rhamnose, xylose, arabinose, lactose, trehalose (Laboratory Reagent Quality; British Drug Houses Ltd.); glucose, soluble starch, lactic acid (British Drug Houses Ltd., Analar chemicals); dihydroxyacetone, ribose, raffinose, glucosamine hydrochloride (L. Light & Co.); maltose (Hopkin & Williams; and Thomas Kerfoot & Co., Biochemical Reagent quality). In view of the importance of the cellobiose fermentation three different specimens of this sugar were employed (British Drug Houses, Ltd.; L. Light & Co.; Thomas Kerfoot & Co., Biochemical Reagent quality). The sucrose used was ordinary household sugar.

One specimen of bacterial levan was newly prepared by growing *Bacillus megatherium* A.T.C.C. 697 on the liquid sucrose medium of Tarr & Hibbert

(1931). It was purified by two ethanol precipitations and yielded less than 1 % of glucose on hydrolysis. Another specimen, at least 8 years old and prepared similarly, was also available. Purified inulin was a gift from the Edinburgh University Chemical Department.

RESULTS

Evidence in favour of regarding the 'iodophilic polysaccharide granules' of rumen protozoa as starch. According to Pucher *et al.* (1948) the iodine complex of starch, but not that of glycogen, is precipitated in presence of 6 % sodium chloride. They extracted the polysaccharides from plant and animal material by the perchloric acid method of Nielsen (1943). By means of these procedures the best dried protozoan polysaccharide granule preparations obtained previously (Oxford, 1951) were found to contain at least 70 %, the granule-filled protozoa (*c.* 1 % N content) at least 60 %, and other protozoan preparations (*c.* 4 and 5 % N) 50 and 40 %, respectively, of 'starch' in the above sense. It is, therefore, justifiable to refer tentatively to the protozoan storage polysaccharide as starch, rather than glycogen, although a final decision must await the results of detailed structural studies now being carried out elsewhere. As shown later, both holotrichs and oligotrichs contain 'starch' in the Pucher *et al.* sense of the term.

Preparation of holotrich starch in quantity by fermentation of glucose by whole rumen liquor. This experiment continued for three months, with withdrawal of rumen contents (1 l.) from each of two fistulated sheep three times a week. The muslin-strained rumen liquid was used directly as inoculum, and starch granules were liberated from the holotrich ciliates in the washed protozoan white bottom deposits left after glucose fermentation, by the action of Teepol. Care was taken to exclude as much protozoan debris and unburst protozoa as possible from the final dried product. The mean yield from 33 separate fermentations was 1·45 g. (extremes 0·4 and 2·2 g.). The lowest yield was obtained in the 9th fermentation, after which the sheep were given one week's rest. Thereafter, the starch yield was well maintained, the final (33rd) yield being 1·56 g. of which 70 % came from the protozoa supplied by sheep No. 272. During the course of this starch accumulation, opportunity occurred of working up the whole rumen contents of two hay-fed sheep killed for another purpose. Each sheep afforded *c.* 3 l. of strained rumen liquor, and the respective holotrich starch yields were 1·6 and 3·6 g. With the kind of meadow hay fed to these sheep, the maximum yield of rumen holotrich starch seems, therefore, to be of the order of 1 g./l. of rumen contents.

Yields of holotrich starch by fermentation of soluble carbohydrates by washed rumen protozoa. The whole range of sugars was first tested by means of washed protozoa obtained from the deposit on the muslin when rumen contents were strained (see under Methods). Less than 1 mg. starch/100 mg. substrate was obtained in the following instances: dihydroxyacetone, xylose, arabinose, ribose, galactose, mannose, rhamnose, glucosamine, maltose, lactose, trehalose, raffinose, soluble starch (as paste), lactate, or when no substrate was provided. These results are regarded as completely negative. The positive results were obtained with glucose, fructose, sucrose, cellobiose, bacterial levan and inulin, and are summarized in Table 1. It will be seen that they vary from 4 to 7 % with cellobiose to 18 to 24 % with fructose, which, as previously noted (Oxford, 1951), appeared to be the best substrate for protozoan starch formation.

Table 1. *Holotrich starch yields when well-washed protozoa fermented soluble carbohydrates*

Carbohydrate fermented	Starch yields in separate experiments (%)	Mean starch yield (%)
Glucose	11, 13, 17, 19, 20, 21	17
Fructose	18, 21, 24	21
Sucrose	6, 11, 14	10·3
Cellobiose	4, 6, 6 (Kerfoot); 7, 7 (B.D.H.); 4 (Light)	5·7
Inulin	21	21
Bacterial levan	10 (old); 19 (new)	14·5

The surprising result that sucrose and cellobiose were the only disaccharides utilized for starch formation was confirmed by using washed protozoa from strained rumen liquor as inoculum. In each experiment two disaccharides were compared side by side or else a disaccharide was compared with a control containing no substrate. The results, summarized in Table 2, in every way confirmed those in Table 1.

Table 2. *Holotrich starch formation from disaccharides*

	Yield of holotrich starch (mg./700 mg. sugar supplied) from					
Sugars compared	Sucrose S	Cellobiose C	Lactose L	Maltose M	Trehalose T	No substrate N
S v. N	330	.	.	.	.	30*
	320	.	.	.	.	40**
S v. C (Kerfoot)	230	133	.	.	.	.
C (Kerfoot) v. L	.	95	5	.	.	.
C (Light) v. M	.	110	.	10	.	.
C (Kerfoot) v. M	.	210	.	40	.	.
S v. T	220	.	.	.	30	.
Mean yield	275	137	5	25	30	35

* Protozoa worked up at end of fermentation period.
** Protozoa worked up at beginning of fermentation period.

In every instance the isolated starch was compared with that obtained by glucose fermentation with respect to granule shape and size, and colour given with iodine. It was evident that the same substance was always obtained, no matter whether the structural unit given as substrate was glucose or fructose, or both.

The starch of rumen oligotrich ciliates. In spite of many experiments made to obtain a suspension of iodophilic oligotrichs unmixed with holotrichs and their starch grains, only one method of limited application has so far been devised, namely, fermentation of glucosamine hydrochloride for 5–6 hr. by washed protozoa. The holotrichs thereby disappeared completely as organized structures, for reasons at present unknown, and when the bottom protozoan layer was isolated and washed by decantation, a greyish (not white) deposit was obtained which consisted chiefly of iodophilic *Diplodinium* with some *Entodinium*. When the fermentation was prolonged overnight the oligotrichs in turn were no longer particularly iodophilic.

An iodophilic preparation so obtained was dried *in vacuo* at room temperature (50 mg. from 1 l. rumen contents) and then submitted to the starch isolation procedure of Pucher *et al.* (1948). A 7·4 % yield of almost white starch was obtained, completely soluble in hot water. It gave a purple colour with iodine, almost indistinguishable from that given by holotrich starch; the oligotrich starch colour was perhaps a shade bluer in tone. The iodophilic oligotrichs were also sufficiently fragile to be burst by Teepol with starch liberation.

Although an increase in the number of iodophilic oligotrich ciliates is plainly revealed by microscopic examination during the course of a glucose or fructose fermentation, there was no such increase when cellobiose was the substrate. Only the holotrichs became filled with starch grains in this instance.

The starch of rumen contents of grass-fed sheep. The sward consisted chiefly of ryegrass and clovers. In all three sheep killed, there was a rich rumen micro-fauna consisting largely of oligotrichs, although holotrichs were also plentiful. The glycogen-containing pseudo-yeasts, and the large starch-containing 'cigar-shaped bacilli' of van der Wath (1948) were also present in great numbers. The isolated 'starch' in Table 3 may not, therefore, consist entirely of ciliate starch, even assuming that no starch from the fodder was present in the rumen. This is probably true for the second sheep killed early in June, but is not so certain for the one killed in August. All specimens of isolated starch gave a purple colour with iodine.

Table 3. *Starch extraction from rumen contents of grass-fed sheep*

Date of slaughter of sheep	Period of grazing (days)	Carcase weight (lb.)	Weight of rumen contents (g.)	Volume of strained rumen liquor (ml.)	Weight of solids (S) by evaporation of strained rumen liquor (g.)	Starch in S by $HClO_4$ extraction (%)
23/5/1950	18	.	5823	2448	. . .	(0·45 g.)*
6/6/1950	32	63	4860	2600	162	0·9 1·05 0·9
1/8/1950	88	56	5330	2450	97·2	0·6 0·6

* By direct isolation of holotrich ciliates and subsequent bursting by Teepol.

DISCUSSION

The main interest of the results on the protozoan fermentation of soluble sugars centres in the ability of the holotrich ciliates to form starch from cellobiose. The fact that the levans are also suitable substrates for starch formation is not so surprising in view of the fact that invertase is known to attack them (cf. Archbold, 1940), and the probability that these protozoa must have a very active invertase, since sucrose appears to be as suitable as glucose with respect to starch formation. It would seem that the holotrichs must contain the enzyme cellobiase (but not, however, maltase), since it is inconceivable that starch can be built up directly from cellobiose. The researches of Hungate (1950) have established a close connexion between cellulose and cellobiose fermentation by rumen bacteria, so that if it be accepted that rumen protozoa can also attack cellulose (i.e. by ingestion followed by enzymic digestion of plant particles) it follows that they ought to be able to attack cellobiose. Unfortunately for this line of reasoning, those ciliated protozoa with very small mouths which ought not, and by microscopic examination clearly do not, ingest plant particles to any extent, namely, the holotrich ciliates, are the very ones which appear to contain cellobiase, whereas the oligotrich ciliates, which are very active in ingesting plant particles, do not appear to be able to utilize cellobiose for the formation of their starch-like reserve polysaccharide. The alternative hypothesis, that when the cellulose of plant particles in the rumen is being attacked by bacteria, there may be local pockets of soluble sugars including cellobiose near these particles, would, therefore, appear to merit further consideration. The extreme motility and water-passing properties of the holotrich ciliates would at least enable them to take advantage of suitable soluble sugars to be found anywhere in the rumen contents, provided bacterial competition were not too great. As shown previously (Oxford, 1951) this competition is not too great when glucose, fructose or sucrose is fermented but must be serious in the case of cellobiose since no appreciable starch formation by holotrich ciliates took place when that sugar was fermented by whole rumen liquor. It is only when the protozoa are freed as far as possible from bacteria that their utilization of cellobiose can be demonstrated. Even then, however, the other disaccharides tested are useless to the protozoa. The fact that maltose appears to be of no use to the protozoa for starch formation is surprising, and may indicate that the well-known phosphorylating mechanism for starch production is active here also. Our observations certainly do not support the claims of Trier (1926) or of Wieneck (1934) that lactose is converted into 'glycogen' by rumen ciliates, nor those of van der Wath (1942) that maltose is similarly utilized.

In view of the activity of the holotrich ciliates in converting bacterial levan and inulin into starch *in vitro*, it is surprising that the rumens of sheep fed on Spring grass, which is known to contain far more soluble sugars than hay and in particular soluble levans (see Norman, 1936), do not contain more holotrich starch than the small amounts reported in Table 3. Spring grass, being rich in soluble protein also, would appear to encourage the growth of the oligotrich ciliates preferentially, a fact already noted by Mangold (1929). With hay-fed sheep, however, the rumen holotrich ciliates become of relatively greater importance, and a clear indication of the suitability of this environment for them lies in the fact that even when a volume of rumen contents equal to the whole is withdrawn in three equal instalments during the course of a week, the holotrich population remains approximately constant over many months, as shown by the starch yields obtained during glucose fermentation by strained rumen liquor *in vitro*.

It may be emphasized that one purpose of this paper has been to show that, from a biochemical point of view, it is inadvisable to group all the rumen ciliates together with respect to their biochemical activities. For example in the paper by Burroughs, Frank, Gerlaugh & Bethke (1950) on cellulose fermentation by rumen contents *in vitro*, it is vaguely stated that some of the larger types of protozoa failed to survive throughout the entire series of fermentations. Since the two great groups of rumen ciliates (i.e. holotrich and oligotrich) are easily distinguished in unstained preparations, even with the 2/3 in. objective, it is to be hoped that a distinction will henceforward be made between them in all such fermentation studies with rumen contents.

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The Role of Certain Electrolytes in the Adsorption of Staphylococcal Bacteriophages

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SUMMARY: The role of electrolytes in the adsorption of the citrate-sensitive staphylococcal typing-phages and the citrate-insensitive phage K on to their propagating strains was studied in broth + Na citrate and in distilled water + $CaCl_2$, $MgCl_2$, and NaCl. Some phages when in the free state were partially inactivated by 1% (w/v) Na citrate. In all except two phages, K and 51, citrate inhibited adsorption in broth; adsorption occurred in distilled water. Three phages, 7, 42B and 42E, had a specific requirement of 50 μg. $CaCl_2$/ml. for adsorption. With the remainder, the requirements of $CaCl_2$ varied from 5 to 400 μg./ml.; similar amounts of $MgCl_2$ acted equally well.

With the citrate-sensitive phages adsorption in NaCl was variable and, where it occurred, recovery of viable phage from cells infected in NaCl was less than from cells infected in $CaCl_2$. These phages appear to have a specific requirement of divalent cations for adsorption, suggesting that adsorption and penetration are enzymic in character.

The electrolyte requirements of the citrate-insensitive phage K were related geometrically to the valence of the cations used, 5 μg. $CaCl_2$ or $MgCl_2$/ml. or 25 μg. NaCl/ml. giving optimum adsorption.

Early in the study of bacteriophage phenomena it was observed that citrate or oxalate in the media prevented multiplication of certain phages. On the basis of such observations Burnet (1933) proposed that bacteriophages should be divided into 'citrate-sensitive' or '-insensitive', and showed that this sub-division was of value in the classification of phages which lysed intestinal bacteria and staphylococci (Burnet & Lush, 1935).

The mode of action whereby citrate and oxalate inhibit phage multiplication has been investigated in detail for only a few phages. The action of these substances is assumed to be due to their de-ionizing effect on calcium and/or magnesium ions, but little information is available on the role which these ions play in phage synthesis (Cohen, 1949). Delbrück (1948) described a mutant of coli phage T4 which required Ca^{++} as well as tryptophan for adsorption. On the other hand, Adams (1949) showed that coli phage T5 required calcium for growth but not for adsorption. The same was apparently true for the citrate-sensitive coli phage of Elford & Andrewes (1932), coli phage T1 (Puck, 1949), and a staphylococcal phage, 51 (Rountree, 1947). At the same time, citrate may inactivate free phage, although Adams (1949) showed that, with the coli phages, inactivation in salt solutions is due to Na ions rather than to citrate.

The present work studies the point in the staphylococcal phage cycle at which citrate acts and was concerned in the first instance with its effect on free phage and on adsorption. From this point of departure the role of Ca, Mg and Na ions in phage adsorption has been studied.

MATERIAL AND METHODS

The typing phages of Wilson & Atkinson (1945) and phage K and their respective propagating strains were used. Certain of their characteristics have been already described (Rountree, 1949). In the adsorption tests, specific adsorption of phages on to their sensitive propagating strains was studied.

All glassware was carefully cleaned in chromic-sulphuric acid mixture and well rinsed in distilled water. Distilled water (redistilled in glass) was used to make the salt solutions and, where required, to suspend bacterial cells and to dilute phages.

Phage counts were made on plates surface-inoculated with the propagating strain, each plate accommodating four separate inocula of 0·01 ml. The media used were glucose (0·15 %) veal broth and the same broth plus 1·2 % shredded agar and 0·01 % $CaCl_2$.

Adsorption tests were carried out in tubes 80 mm. long × 10 mm. diam., the final volume of reactants in each tube being 1 ml.

EXPERIMENTAL

Sensitivity of free phage to sodium citrate

The phages were tested for sensitivity to 1 % (w/v) Na citrate in broth in absence of bacterial cells. The phages were diluted in broth so that the final dilution (in broth + citrate) would give an estimated count of 100–200 plaques. The results were expressed as per cent inactivation after 20 min. in a water-bath at 37° as compared with controls in broth alone. It was considered that the dilution of citrate which took place when the 0·01 ml. inoculum was spread on the agar plate was adequate to prevent any interference in adsorption of phage on to the bacterial cells.

Different batches of nutrient broth may contain variable amounts of ionizable calcium and it has been recognized (Williams Smith, 1948) that a low Ca^{++} content may render certain batches of media unsuitable for phage growth. Unfortunately no chemical method of estimating ionized calcium is available, so no very precise information can be obtained on the concentrations of citrate which inactivate free phage in broth. However, a number of estimations made with different batches of broth and the same phages showed good agreement (Table 1).

Those phages which were almost completely inactivated by citrate belong to serological group B, which has been shown (Rountree, 1949) to be markedly unstable to heat and storage. The correlation between serological type and sensitivity of free phage to citrate is not, however, absolute, since phages 29A, 31A, 42D and 44 also belong to this serological group. Since the broth in which these tests were made contained 0·5 % NaCl, and no inactivation of

Table 1. *Percentage inactivation of free phage in 1 % Na citrate broth in 20 min. at 37°*

Inactivation (%)	Phages
Nil	44, 51, K
10	42E
30–35	6, 7, 42D
45–60	3A, 3B, 29A, 31A, 42B
70–85	3C, 47, 47A, 47B, 47C
90–98	29, 31, 31B, 42C, 52, 52A

phage occurred in broth at 37° in the absence of citrate, it was concluded that the inactivating effect of the Na citrate was due to the citrate radical and not to Na ions.

Inhibition of phage adsorption in the presence of citrate

Sufficient Na citrate was added to overnight broth cultures of the appropriate propagating strain to give final concentrations of 0·1, 0·5 or 1·0 % (w/v) Na citrate. Phage was then added, and the mixtures incubated at 37° for 20 min., centrifuged (angle centrifuge), and the free phage remaining in the supernatant determined. Controls containing no bacterial cells were included in each test.

With phages K and 51 there was no inhibition of adsorption in the presence of 1 % citrate. Adsorption of phages 3A, 3B, 3C, 6, 7, 42B, 42D, 42E, 44, 47, 47A, 47B and 47C was inhibited by 0·5 % Na citrate. With the more unstable phages 31, 31B, 42C, 52 and 52A, it was difficult to evaluate the results since almost as much phage was inactivated in the broth and citrate controls as was adsorbed by the cells in absence of citrate. However, with phages 29 and 29A definite evidence of inhibition of adsorption by 0·5 % citrate was obtained. Table 2 gives the result of a typical experiment.

Table 2. *Inhibition of adsorption of phage* 42 E *by* 1 % *Na citrate*

Tube No.	Contents	Plaque count of supernatant
1	Cells+phage	11; 9*
2	Cells+phage+Na citrate 1 %	300; 322
3	Broth+phage	418; 400
4	Broth+phage+Na citrate 1 %	370; 340

* In this and subsequent tables, duplicate plaque counts are presented.

Adsorption of phage in the presence of electrolytes

Calcium chloride, magnesium chloride and sodium chloride were used in tests designed to demonstrate their effect on phage adsorption. The adsorbing cells were centrifuged from 5- or 18-hr. broth cultures and resuspended in distilled water at a concentration of approximately 10^9 cells/ml. The use of high titre phages diluted in distilled water ensured that the phage suspensions contained negligible amounts of electrolytes derived from the medium used for preparing the phage filtrates. The salt solutions were made up in varying strengths so that addition of 0·1 ml. volumes to the adsorption tubes would result in the final concentrations required. After incubation of the mixtures in a water-bath at 37° for 20 min. the tubes were centrifuged and plaque counts made on the supernatant fluid. Some phages were unstable in simple salt solutions and in such cases it was necessary to test the centrifuged cells for the presence of viable adsorbed phage by removing the supernatant fluid, resuspending the cells in the distilled water and then plating. The minimum concentration of each salt which permitted 90 % adsorption was determined for each phage. No adsorption of phage was observed in phage + cell mixtures lacking electrolytes.

Table 3. *Adsorption of phage* 42 E *in presence of electrolytes*

Quantities of electrolytes shown in μg./ml. solution.

Tube No.	Contents Cells + phage in	Plaque count of supernatant	Adsorption (%)
1	Distilled water	140; 148	0
2	$CaCl_2$ 10	134; 141	0
3	20	100; 60	40
4	50	6; 3	97
5	100	4; 0	98
6	150	3; 5	97
7	200	1; 2	99
8	$MgCl_2$ 200	103; 91	0
9	NaCl 100	103; 99	0
10	800	94; 82	0

	Controls (phage only)		Plaque count	
			Time 0	Time 20 min.
11	Distilled water		143; 131	119; 142
12	$CaCl_2$	200	132; 112	124; 96
13	$MgCl_2$		157; 152	84; 112
14	NaCl	800	138; 120	116; 124

Table 3 shows the plaque counts obtained in a typical experiment using phage 42 E. The average initial concentration of phage calculated from counts obtained from control tubes 11–14 plated at zero time was used as the base for calculating the percentage adsorption. With phage 42 E, 50 μg. $CaCl_2$/ml. allowed maximum adsorption; $MgCl_2$ could not replace $CaCl_2$, and NaCl up to 800 μg./ml. was inactive.

The results obtained with 21 phages are given in Table 4. Where no activity could be demonstrated for electrolyte in concentrations up to 1000 μg./ml. a blank has been left in the table. Two phages, 42 C and 44 A, were so unstable in distilled water and salt solutions that no results were obtained. Phages 7, 42 B and 42 E showed a specific requirement of calcium for adsorption, 90 % adsorption occurring in 50 μg. $CaCl_2$/ml. and no adsorption in $MgCl_2$ or NaCl. Phage 47 required 150 μg. $CaCl_2$/ml. for adsorption; little adsorption occurred with a similar concentration of $MgCl_2$ while with 800 μg. NaCl/ml. some adsorption took place.

Table 4. *Minimum concentrations of $CaCl_2$, $MgCl_2$, and NaCl giving 90 % adsorption of various phages in 20 min. at 37°*

Phage	Minimum concentration (μg./ml.) $CaCl_2$	$MgCl_2$	NaCl
K	5	5	25
3A, 3B, 3C	10	10	400
7, 42B, 42E	50	—	—
6, 47A	20	20	800
29, 52	400	400	—
29A, 52A	100	100	—
31, 31B	150	150	—
42D	50	50	—
44	200	200	—
47	150	—	800
47B	5	5	100
47C	50	50	800
51	50	50	1000*

— No activity demonstrated with 1000 μg./ml.
* Partial adsorption only.

With the remainder of the typing phages, the $CaCl_2$ requirements varied from 5 to 400 μg./ml., and in all 5 to 400 μg. $MgCl_2$/ml. gave the same amount of adsorption. For phage K, which is citrate-insensitive, concentrations of 5 μg. $CaCl_2$ or $MgCl_2$/ml. or 25 μg. NaCl/ml. permitted 90 % adsorption. These results indicate that the adsorption of phage K may be a simple electrolytic phenomenon which can be related to the valence of the cations used. With those citrate-sensitive phages in which adsorption in NaCl could be demonstrated, the relationship between valence and the concentration of electrolyte required for adsorption was not a geometric one. For example, phage 47B required 100 μg. NaCl/ml. compared with 5 μg. $CaCl_2$ or $MgCl_2$/ml. With the phages 29, 29A, 31, 31B, 42D, 44, 52 and 52A no NaCl activity could be demonstrated in concentrations up to 1000 μg./ml.; higher concentrations could not be tested because they inactivated free phage. If the relationship between valence and cation requirements were a simple geometrical one, those phages having high Ca requirements would need almost astronomical amounts of NaCl for adsorption. On the other hand, phage 42D which required 50 μg. $CaCl_2$/ml. might be expected to adsorb, at least partially, on to cells in 1000 μg. NaCl/ml. However, no viable phage could be recovered from such cells.

The adsorption tests also supplied evidence that the requirements of the phages were related to the phage and not to the cell surface. Phages 42B and 47C are both propagated on staphylococcus strain 1163, but whereas phage 47C was adsorbed in 50 μg. $CaCl_2$ or $MgCl_2$/ml., phage 42B showed a specific requirement of 50 μg. $CaCl_2$/ml. Phages 31 and 44 required differing amounts of $CaCl_2$ or $MgCl_2$ for 90 % adsorption on to strain 18. Finally phage K when adsorbed on to strain 145 required only 5 μg. $CaCl_2$/ml. as compared with phage 51, which required 50 μg./ml. for adsorption to this strain.

Estimates of the amount of viable phage recovered from sensitive cells infected with phages 3A, 3B or 3C in various concentrations of $CaCl_2$, $MgCl_2$ or NaCl provided some interesting information. The results obtained with phage 3A are given in Table 5. With optimum amounts of $CaCl_2$ or $MgCl_2$ almost the whole amount of added phage could be recovered from the cells. With 400 μg. NaCl/ml., however, although the amount of free phage present in the supernatant indicated 95 % adsorption, only 25 % of adsorbed phage was recovered. Furthermore, there was no increasing yield of recoverable adsorbed phage with increasing concentrations of NaCl.

Table 5. *Adsorption of phage* 3A *in the presence of various concentrations of $CaCl_2$, $MgCl_2$, and NaCl, showing amounts of unadsorbed phage in the supernatants and of adsorbed phage recovered from the cells*

Quantities of electrolytes in μg./ml. solution.

Tube No.	Contents Cells+phage in		Plaque counts Free phage	Adsorbed phage
1	Distilled water		212; 250	14; 9
2	$CaCl_2$	5	33; 41	152; 121
3		10	5; 4	170; 130
4	$MgCl_2$	10	10; 11	136; 170
5		20	4; 3	158; 210
6	NaCl	100	118; 80	49; 68
7		200	37; 29	32; 43
8		400	9; 8	53; 72
9		800	18; 18	55; 75

These results resemble those obtained by Puck (1949) who found that coli phage T1 diluted in distilled water and adsorbed on to coli cells in the absence of calcium ions could not be recovered. Puck ascribed his finding to 'sensitization' of phage by dilution in distilled water or phosphate buffer, and suggested that this sensitization produces a block in some step in the virus cycle which occurs after adsorption. This step may be that of penetration of the cell as distinct from adsorption on to the cell surface. If this be so, the recovery of viable phage from staphylococcal cells when adsorption has taken place in NaCl might be due to the cells themselves providing sufficient calcium to allow 'activation' and penetration of some adsorbed phage. Since constant amounts of phage 3A were recovered from constant numbers of cells infected in increasing concentrations of NaCl, there is some evidence for this view. Cells grown in broth containing citrate should be lacking in this postulated available calcium and, if infected in NaCl, no phage should be recoverable from them. An experiment was set up in which the propagating strain for phage 3A was grown in 0·5 % citrate broth for 5 hr., the cells washed in distilled water and resuspended therein. From these cells no phage was recovered when adsorption took place in NaCl, while in $CaCl_2$ all the phage adsorbed was recovered; the results of a typical experiment are given in Table 6.

Table 6. *Percentage adsorption and percentage recovery of phage 3A adsorbed on to cells grown in citrate broth and infected in* $CaCl_2$ *or various concentrations of NaCl*

Quantities of electrolytes in μg./ml. solution.

Tube No.	Contents Cells+phage in	Adsorption (%)	Recovery (%)
1	Distilled water	0	0
2	$CaCl_2$ 50	99	100
3	NaCl 100	60	0
4	200	80	0
5	400	90	0
6	800	100	0

DISCUSSION

Phage adsorption was originally regarded as a relatively simple process in which specific receptor structures of phage and cell surfaces were brought together by non-specific electrolytic forces. However, the observations of Anderson (1945) on the tryptophan co-factor requirements of certain coli phages and of Delbrück (1948) on a Ca^{++}-requiring mutant coli phage indicated that the electrolyte theory could no longer be regarded as sufficient and that, besides electrical forces, enzymic reactions involving either phage or cell surface or both must be postulated.

With the staphylococcal phages used here the adsorption process may be equally complex. With two exceptions, adsorption of these phages was inhibited by citrate although adequate amounts of electrolyte were present had the adsorption been a simple electrical phenomenon. With phages 7, 42B and 42E, a specific co-factor requirement for Ca^{++} was demonstrated. With the other phages Mg^{++} could be substituted for Ca^{++}. There was also evidence that each phage had a characteristic Ca^{++} requirement, rather than that the Ca^{++} requirement was related to the nature of the bacterial cell surface. The inactivation of some phages by citrate in broth suggests that citrate may combine with Ca^{++} on the surface of the phage particle and so block the sites involved in adsorption.

With phages K and 51 there was neither inactivation of free phage by citrate nor inhibition of adsorption. The requirements of phage K were directly related to the valence of the ions used. With phage 51 this could not be demonstrated experimentally since the amount of NaCl 'theoretically' required inactivated the phage in absence of the stabilizing action of nutrient broth.

The fact that certain of the phages which had low requirements of divalent cations adsorbed in NaCl does not invalidate the hypothesis that divalent cations, particularly Ca^{++}, are of primary importance in adsorption. There was a lower recovery of viable phage from cells infected in NaCl, and furthermore there was complete loss of adsorbed phage when the cells had been previously grown in citrate broth. This suggests that under certain conditions the cell itself may supply the required Ca^{++} or Mg^{++} and NaCl may merely provide the electrical conditions necessary for apposition of cell and phage. The experimental results, therefore, suggest that Ca^{++} is required for penetration, but they do not exclude the possibility that this is also involved in an early step in the growth cycle following penetration of the cell.

The conclusion that Ca^{++} is important in phage adsorption is of interest in the light of the findings of Burnet and his associates on the importance of Ca^{++} in the reactions of influenza virus, of the receptor-destroying enzyme of the cholera vibrio with erythrocyte surfaces, and the mucoid inhibitors of haemagglutination (Edney, 1949). The adsorption phenomena involved in these reactions appear to be enzymic in nature. The present observations with staphylococcal phages suggest that, at least for some of them, adsorption and penetration may also be enzymic.

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The Distribution of Glutamic Acid Decarboxylase in the Family Enterobacteriaceae, examined by a Simple Chromatographic Method

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SUMMARY: By a simple chromatographic technique with filter-paper disks the distribution of glutamic acid decarboxylase in the family Enterobacteriaceae was surveyed. The decarboxylase occurred irregularly among 230 strains belonging to this family. The test for glutamic acid decarboxylase may prove to be of value in the classification of organisms related to the genus *Salmonella*, in the classification of paracolon types and in the identification of sub-groups within the genera *Escherichia* and *Aerobacter*.

We have shown (Proom & Woiwod, 1949) that when bacteria are grown on an acid-hydrolysed casein medium changes may occur in the culture fluids, revealed chromatographically, which are peculiar to certain species or groups of bacteria; and we suggested that they might be of value in bacterial classification. We have now surveyed the distribution of glutamic acid decarboxylase (GAD) among strains and species of the family Enterobacteriaceae and have devised a very simple chromatographic technique for demonstrating its presence.

METHODS

The production, by some strains of bacteria, of γ-aminobutyric acid by specific decarboxylation of glutamic acid can be demonstrated by the chromatographic technique of Woiwod (1949). It was apparent, however, that for routine use in a bacteriological laboratory a much simpler method was required, and a filter-paper disk technique was devised which is a considerable modification of that described by Rutter (1950).

Filter-paper disk chromatography

The apparatus for the filter-paper disk chromatographic method is illustrated in Fig. 1 and the method is as follows. The lid (A) of a large Petri dish (*c.* 14 cm. diam.) is placed on the bench with its edges uppermost, and in it is placed a sheet of filter paper (F), which covers the bottom and is saturated with water. Centrally on the wet filter paper is placed the lid (B) of a smaller Petri dish (*c.* 9 cm. diam.) containing a layer (D) of *m*-cresol saturated with water. This inner dish (B) can be covered by the bottom of the larger Petri dish (G). Provided the filter paper is kept saturated with water the *m*-cresol/water equilibrium is maintained in the chamber formed by the two parts A and G of the larger Petri dish.

The chromatogram disks used are 12·5 cm. Whatman No. 4 filter papers. A paper is marked with a pencil, eight marks being made and numbered, equidistant around the circumference of a circle of radius 1 cm. from the centre of the paper. This can be conveniently done with the aid of a circular plastic disk of the same diameter as the filter paper and with holes drilled at the appropriate positions.

A small hole is made in the centre of the filter paper (C) to take a 'wick' (E) made of a loosely-rolled cylinder of Whatman No. 4 paper cut to the required length. The samples to be chromatographed are spotted on to the filter-paper disk, at the marked positions, using a fine glass capillary (Woiwod, 1949). The filter-paper wick (E) is then pushed through the hole in the centre of the paper disk (C) and the disk placed on the top of the smaller Petri dish (B), ensuring that the wick dips into the layer (D) of *m*-cresol. The Petri dish (B) and disk (C) are then covered with the inverted bottom (G) of the larger Petri dish. The *m*-cresol is drawn up the wick by capillarity and resolution of the constituents of the sample occurs as the solvent front moves as an expanding circle across the filter-paper disk.

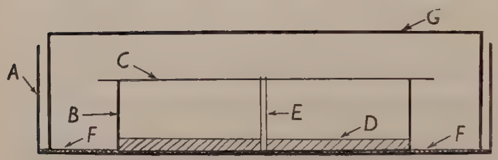

Fig. 1

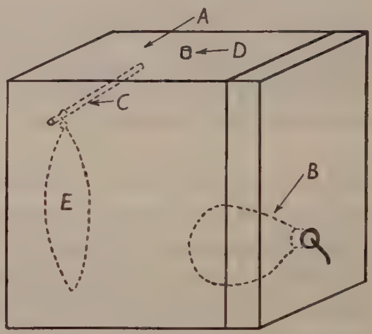

Fig. 2

Fig. 1. Diagram of apparatus for filter-paper disk chromatography. A, Inverted 14 cm. Petri lid; B, 9 cm. Petri dish; C, Filter-paper disk; D, *m*-cresol saturated with water; E, Filter-paper wick; F, Filter paper saturated with water; G, Inverted bottom of larger Petri dish.

Fig. 2. Diagram of biscuit-tin drying oven. A, 9-in. cube biscuit tin; B, Electric lamp fixed in lid; C, Rod for suspending filter papers; D, Outlet for connexion to water pump; E, Suspended filter paper.

The apparatus is left on the bench at room temperature. It takes about 1 hr. for the solvent front to reach the circumference of the filter-paper disk. The filter-paper disk is then removed by a clip attached to the edge of the paper and is suspended and dried in a hot-air oven. The oven can be simply made from a biscuit tin (A), Fig. 2 (*c.* 9 in. cube) heated with a 60-W electric lamp; the fumes are removed from the tin by a rubber tube connected to a water pump. Such an apparatus is illustrated in Fig. 2 and was satisfactory in routine use. After drying in the oven, which takes about 30 min., the disks are dipped in a solution of 0·1 % (w/v) ninhydrin in chloroform and are heated for a few minutes in the oven to develop the chromatogram. Very occasionally a batch of filter papers is encountered in which the solvent front moves in an irregular fashion from the centre of the paper. Although it is usually possible to obtain results from such papers they are not satisfactory.

Preparation of cultures for chromatography

The culture medium used consisted of (in %, w/v): glutamic acid, 1; glucose, 3; sodium chloride, 0·5; with added metallic salts and growth factors as in the medium of Proom & Woiwod (1949). The growth factors and metallic salts were not necessary for the majority of the strains examined but were included for the benefit of fastidious organisms. The medium was adjusted to pH 7·4, dispensed in 2 ml. amounts in 1/4 oz. screw-capped bottles and sterilized by autoclaving at 15 lb. for 30 min. These bottles were inoculated with strains to be tested and incubated for 24 hr. at 37°, or at 28° for those species which grew either poorly or not at all at 37°. After 24 hr. incubation a few drops of Universal indicator (British Drug Houses Ltd.) were added to each bottle and the pH adjusted to about 4·5 with sterile N-HCl. To each bottle was then added 0·5 ml. of sterile 0·2 M acetate buffer (pH 4·5) and the cultures incubated for a further 24 hr. The cultures were then steamed for 1 hr. and tested for the presence of γ-aminobutyric acid by the chromatographic method just described.

Examination of culture fluids for glutamic acid decarboxylase

Glutamic acid does not move appreciably from the starting position and γ-aminobutyric acid moves close to the edge of the filter-paper disk. A positive GAD reaction, therefore, is the appearance of a spot, when developed with ninhydrin, near the circumference, indicating the presence of glutamic acid decarboxylase. Cultures of known positive and negative strains or a solution of γ-aminobutyric acid may be included as controls. After a little experience with the test these controls may be omitted. Since the solvent front moves as an expanding circle across the filter paper the γ-aminobutyric acid spot is not circular but appears as a band 1–2 cm. in width. The results obtained with the filter-paper disk method agree completely with those obtained when the same cultures were analysed on larger papers by the method of Woiwod (1949); the two methods are equally sensitive. Provided a culture shows visible growth in 24 hr. it is not necessary to increase the incubation period either for growth or enzyme action at pH 4·5. Occasionally a strain fails to grow in 24 hr. and further incubation is necessary.

RESULTS

The strains examined were from the Wellcome Bacterial Collection supplemented by strains from the National Collection of Type Cultures, the Metropolitan Water Board and the Water Pollution Research Laboratories. Many of the strains from the NCTC were received under names having little more than historical interest and in order to simplify the expression of the results the *coli* and *aerogenes* strains were classified according to their biochemical reactions, using the nomenclature usually employed in the bacteriological examination of water (see *The Bacteriological Examination of Water Supplies*, Report 71, H.M. Stationery Office, 1939). The paracolon organisms were regarded as variants, being non-fermenters or late-fermenters of lactose, of a parent species; for example strains received under the name of *Bacterium columbense* were classified as non-lactose-fermenting variants of *Bact. coli* type I.

Table 1. *The distribution of glutamic acid decarboxylase in the family Enterobacteriaceae*

Organism	Reactions				Glutamic acid decarboxylase test. No. of strains		
	M.R.	V.P.	Citrate	Indole	Examined	Positive	Negative
Bact. coli type I	+	−	−	+	72	71	1
Bact. coli type II	+	−	−	−	1	0	1
Bact. coli intermediate type I	+	−	+	−	12	0	12
Bact. coli intermediate type II	+	−	+	+	3	2	1
Bact. coli irregular type I	+	−	−	+	3	3	0
Bact. aerogenes type I	−	+	+	−	16	0	16
Bact. aerogenes type II	−	+	+	+	5	2	3
Bact. cloacae	−	+	+	−	4	0	4
Friedländer's bacillus					9	0	9
Paracolon sp. (*Bact. coli* type I except for lactose)					11	11	0
Paracolon sp. (*Bact. coli* intermediate type I except for lactose)					4	0	4
Paracolon sp. (*Bact. aerogenes* type I except for lactose)					4	0	4
Plant pathogens (*Bacterium* (*Erwinia*) sp.)					6	0	6
Serratia sp.					7	0	7
Salmonella sp.					43	0	43
'Arizona' group of coliforms					6	0	6
Shigella sp.					24	18	6

M.R., Methyl red test; V.P., Voges-Proskauer test.

The results are summarized in Table 1. Of 72 strains of *Bact. coli* type I and 3 strains of *Bact. coli* irregular type I, all except one strain of *Bact. coli* type I gave a positive GAD test. All these strains were of animal origin (human, ovine, bovine, canine), most of the bovine strains having been isolated from cases of white scour. The correlation between the presence of GAD and the animal origin of the strain is, in this group, nearly complete and rather better than the correlation with the results of the Eijkman reaction.

All of 12 strains of *Bact. coli* intermediate type I, 16 strains of *Bact. aerogenes* type I, and 4 strains of *Bact. cloacae* were GAD negative.

With the few strains available of *Bact. coli* intermediate type II and *Bact. aerogenes* type II the GAD test was variable. There was an interesting relation between the GAD reaction of the non-lactose-fermenting or paracolon variants, and of the parent strains. Thus 11 strains which had the biochemical reactions of *Bact. coli* type I, except for delayed or no reaction with lactose, were GAD positive whereas 4 non-lactose-fermenting variants of *Bact. aerogenes* type I and 4 non-lactose-fermenting variants of *Bact. coli* intermediate type I, were all negative.

Nine strains of Friedländer's bacillus, including representatives of serological types A, B and C, were all GAD negative. The biochemical reactions of these strains were variable and none of them had the biochemical pattern of a species which is predominantly GAD positive.

Six strains of plant pathogens (2 strains of *Bact. carotovorum* and 1 strain each of *Bact. amylovorum*, *Bact. salicis*, *Bact. tumefaciens* and *Bact. rhizogenes*) and 7 chromogenic actively-proteolytic strains belonging to the genus *Serratia*, were all GAD negative.

Nineteen strains of *Proteus*, including representatives of the species *Pr. vulgaris*, *Pr. mirabilis*, *Pr. morganii*, *Pr. rettgeri* and a number of X19 and XK strains were either weakly GAD positive or negative and there was no correlation between the GAD reaction and a particular species.

The GAD reaction was also variable in the genus *Shigella*. Twenty-four strains were examined; some were strongly positive, others weakly positive or negative and there appeared to be no correlation between the GAD reaction and the particular species.

Forty-three strains of the genus *Salmonella* were tested and were all negative. Six strains of the closely related 'Arizona' group of coliforms were also negative. Four strains of the 'Bethesda' group of coliforms were also negative but the biochemical reactions of these four strains (NCTC Nos. 7820, 7822, 7824, 7829) would suggest a relationship with *Bact. coli* intermediate type I, a group which is essentially GAD negative.

DISCUSSION

In previous work (Proom & Woiwod, 1949) we showed that when bacteria are grown on an acid-hydrolysate of casein medium changes may be seen on the filtrate chromatograms which in some instances were associated with a bacterial group or species. However, before the chromatographic method can be widely used in routine bacteriology the technique should be simple, and it must be possible to test a number of cultures in a short time, with apparatus which is readily available and occupies little space. Within the limitation that it is only applicable to some simple mixtures of amino-acids our method satisfies these conditions. Results are obtained on the second day after inoculation; the setting-up of the chromatograms for 50–100 cultures occupies about 30 min. and the analysis is completed in about 3 hr.

The biochemical reactions used in the identification and classification of bacteria have, in the main, been selected empirically. There seems no reason to suppose, for example, that the decarboxylation products of amino-acids are of greater or less value in classification than the fermentation of particular carbohydrates. The overriding principles governing the use, for classification, of a particular biochemical reaction are its utilitarian value within the group studied and the ease with which the test can be performed.

From the present results it is apparent that glutamic acid decarboxylase is scattered irregularly among strains and species in the family Enterobacteriaceae as a whole, but that in a few cases the test may be of taxonomic interest.

Sufficient strains of *Salmonella* have been examined to suggest that GAD is not present in members of this genus. Closely related organisms such as the 'Arizona' group of coliforms are also GAD negative. The test may thus be of value in classification of organisms possibly related to *Salmonella.*

The GAD test may also be of value in the classification of the paracolon organisms. If paracolon organisms are regarded as late- or non-lactose-fermenting variants of a parent species our results would suggest that when the parent species is GAD positive the variant would have the corresponding character and vice versa.

King & Fletcher (1950) examined 47 strains of coliform organisms and found that GAD was characteristic of *Bact. coli* type I; they also found a positive correlation between the Eijkman and GAD reactions. We have extended their observations, confirming that *Bact. coli* type I is essentially GAD positive. We have, however, observed the presence of GAD in a minority of strains of *Bact. coli* of other types. The 3 strains of *Bact. coli* irregular type I which were available and 2 of 3 strains of *Bact. coli* intermediate type II, were positive. None of these strains gave a positive Eijkman reaction.

In our hands a few of the strains of *Bact. aerogenes* type II gave a positive Eijkman reaction and in the case of the three strains of *Bact. aerogenes* type II giving a positive Eijkman reaction this was correlated with a positive GAD reaction. The Eijkman tests were performed in MacConkey bile salt lactose broth and incubated in a thermostatically controlled water-bath at 44°, the tests being adequately controlled. We appreciate that the occurrence of Eijkman positive strains in Britain is unusual. However, the positive strains were retested on a number of occasions and the results confirmed.

The results suggest that the relatively simple GAD test may be of value in laboratories routinely testing large numbers of coliform organisms.

We are grateful to Dr E. Windle Taylor and Dr L. A. Allen for some of the strains used in this investigation. Our thanks are due to Miss S. B. Marshall for performing the Eijkman tests and to Mr E. Harris for technical assistance.

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Factors Influencing the *in vitro* Production of Staphylococcal Coagulase

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SUMMARY: The amount of coagulase formed *in vitro* by coagulase-positive staphylococci depends on: the age of the bacterial cells, coagulase being produced only during the latter part of the lag-phase of growth; the period of incubation of the bacterial cells under ideal conditions, the optimum period depending upon the age of the cells when incubation starts; the initial concentration of bacteria. It also depends on the presence in the medium of a fermentable sugar and a factor found in brain-heart infusion and partially replaceable by peptone; the salt concentration in the medium; the initial pH value of the medium; the temperature of incubation: and upon substances present in rabbit serum and, to a lesser extent, horse serum, human serum and egg yolk, egg white and commercial nucleic acid not showing this effect. The activity of rabbit serum is destroyed by treatment with pepsin. Provided that the requisite conditions are met, cell-free coagulase which clots rabbit plasma within a few minutes, can be prepared in less than one hour.

Since the demonstration that coagulase is an important factor in determining the pathogenicity of staphylococci, many papers on this substance have appeared (e.g. Christie & Keogh, 1940; Fairbrother, 1940; Fisher, 1936; Spink & Vivino, 1942; Walston, 1935). Following the lead of Smith & Hale (1944), most workers have attempted to elucidate the mechanism of the reaction of coagulase with fibrinogen (Kaplan & Spink, 1948; Miale, 1949; Tager, 1948*a*; Tager & Hales, 1948). Attempts have also been made to purify coagulase (Tager, 1948*b*; Walker, Derow & Schaffer, 1948) but little attention has been given to the way in which the staphylococci produce coagulase. With a view to investigating the contribution of coagulase to the virulence of staphylococci we have studied the factors which govern its production *in vitro.*

The terminology of the coagulase phenomenon has become confused. We have chosen the simplest names which are in common use. The active principle produced by the organisms in broth is referred to as 'pro-coagulase'. This, together with a substance found in certain sera 'coagulase-reacting factor', forms 'coagulase' which transforms fibrinogen into fibrin.

EXPERIMENTAL METHODS

Inoculum. A 24-hr. culture of *Staphylococcus aureus* (various strains were used) in Wright's broth (Wright, 1933) was centrifuged and the cells suspended in sterile 0·85 % saline. Large clumps of cells were broken up by shaking with glass beads and the strength of the suspension determined by comparison with standard opacity tubes.

Production of coagulase. The inoculum was added to the production-medium, both having been previously warmed to 37°. The mixture was incubated at 37° for the desired period and then thiomersalate was added to give a final concentration of 0·01 %. Coagulase, if present, was contained in the supernatant formed after centrifuging at 4,000 r.p.m. for 10 min. This procedure provided a clear liquid from which over 99 % of the bacterial cells had been removed.

Assay of coagulase. One-tenth ml. of the supernatant was mixed in 3 in. × 3/8 in. tubes with 0·9 ml. of 10 % (v/v) rabbit plasma in saline. The tubes were incubated in a thermostatically controlled water-bath at 37°. The degree of clotting was recorded at intervals as follows: 0=No clot, liquid clear; 1=trace clot, liquid clear; 2=50 % clot, liquid slightly turbid; 3=almost complete clot; 4=complete clot.

An arbitrary unit of coagulase activity was derived from the expression $Kt=100$, where t is the time in min. for a '2' clot to form under the conditions defined above.

RESULTS

In the usual method of testing for coagulase production by staphylococci a portion of broth culture is added to diluted plasma (Fisk, 1940; Williams & Harper, 1946). When the supernatant from this culture is used instead of the whole culture it can be shown that it contains only a small proportion of the total pro-coagulase. A little more pro-coagulase is obtained in the first saline-washing of the cells but none in subsequent washings. The washed cells, however, clot the plasma at about the same titre as the original culture (Table 1).

Table 1. *Production of pro-coagulase by washed staphylococci*

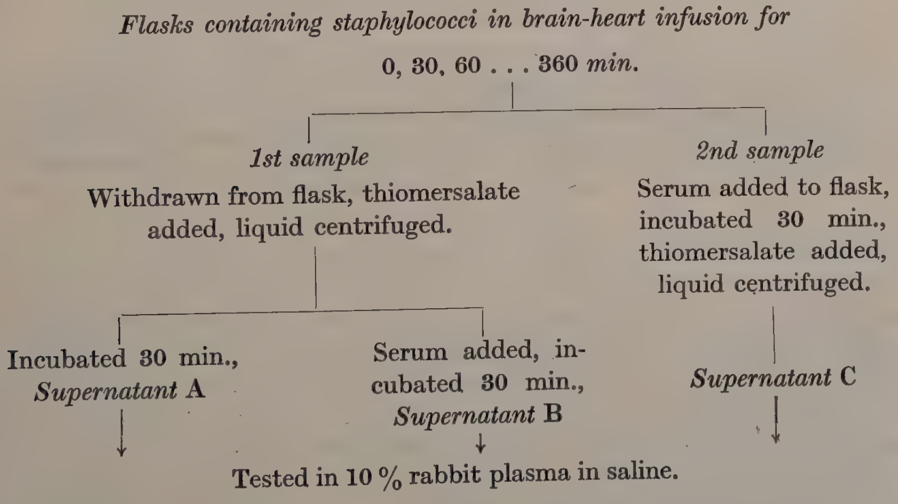
	Highest dilution giving '2' clot		
Source of pro-coagulase	1 hr. reading	2 hr. reading	3 hr. reading
Whole culture	1/24	1/48	1/192
Supernatant	1/6	1/12	1/12
1st washing	—— No coagulase at 1/12 ——		
2nd washing	—— No coagulase at 1/12 ——		
Washed cells	1/24	1/24	1/192

We found that thiomersalate (0·01 %) inhibited the production of pro-coagulase by the washed cells but had no action on the pro-coagulase in the supernatant or on the coagulase formed from this pro-coagulase in the presence of plasma. Thiomersalate was, therefore, used in subsequent experiments whenever we wanted to stop the production of pro-coagulase without affecting its action or that of coagulase. It was apparent from the experiments shown in Table 1 that, under the conditions described, the staphylococci were producing pro-coagulase within a very short time, possibly before the cells started to divide. The rapid production of pro-coagulase was proved by the following experiment. A concentrated suspension of staphylococcal cells (strain BB) was prepared by suspending the washed cells from ten 10 ml. 24-hr. broth cultures in 7 ml. of saline. One ml. of this suspension was added to each of 9 sterile 50 ml. conical Pyrex flasks: 3 containing 10 ml. of Difco brain-heart infusion, 3 containing 9 ml. of this infusion and 1 ml. of rabbit plasma, and 3 containing 1 ml. of the plasma and 9 ml. of saline buffered to pH 7·6. After 1, 2 and 2½ hr. incubation at 37°, one flask of each set was withdrawn from the incubator, the contents centrifuged and thiomersalate added to the supernatants. The coagulase titre of the supernatants against 10 % rabbit plasma in saline was then determined.

The results showed that both broth and plasma must be present for rapid production of coagulase. The supernatant from the complete medium, which had been incubated for only 1 hr., clotted the plasma in 2 hr. at a dilution of 1/192, supernatants from the media lacking either broth or plasma, and incubated for 2½ hr., failed to clot the plasma in 2 hr. at a dilution of 1/6. The active supernatants contained coagulase rather than pro-coagulase since they clotted both plasma and solutions of pure fibrinogen at about the same rate. Living cultures did not clot the fibrinogen solution. The rabbit plasma in the above medium could be replaced by rabbit serum but not by bovine fibrinogen. These experiments indicated that there are two essential factors or groups of factors for the production of coagulase. One is present in rabbit serum and the other in brain-heart infusion.

Experiments on the serum-factor necessary for coagulase production

In order to investigate the effect of serum on bacteria of varying age, the experiment summarized in the following diagram was performed:



Flasks containing staphylococci in brain-heart infusion for

0, 30, 60 . . . 360 *min.*

1st sample

Withdrawn from flask, thiomersalate added, liquid centrifuged.

2nd sample

Serum added to flask, incubated 30 min., thiomersalate added, liquid centrifuged.

Incubated 30 min., *Supernatant* A

Serum added, incubated 30 min., *Supernatant* B

Supernatant C

Tested in 10 % rabbit plasma in saline.

We thus had three supernatants from each flask: A, containing pro-coagulase produced in the absence of serum; B, containing coagulase formed by the action of serum on the cell-free pro-coagulase in A; C, containing coagulase formed by the action of serum on the living cells.

Table 2 and Fig. 1 show that pro-coagulase was first detectable in supernatant A when the cocci had been incubated for 60 min. The amount of pro-coagulase was small (less than 10 units/ml.) and increased to only 45 units/ml. after incubation for a further 300 min. When 10 % of rabbit serum was added at the start of incubation, a large amount (143 units/ml.) of coagulase was produced within 30 min. The results were similar when serum was added to cocci which had been incubated for 30 min. However, cocci incubated for 60 min. before the addition of serum produced only 84 units coagulase/ml. in the subsequent 30 min. and those incubated for 120 min. produced only 29 units/ml., an amount corresponding to the pro-coagulase formed in the same time by cocci which had not been acted on by serum (column C, Table 2). Contact between serum and living bacteria was essential for coagulase production; serum added to the cell-free supernatant for 30 min. produced coagulase in amount corresponding to the pro-coagulase already present (column B). Measurements of pH values and determinations of cell concentrations indicated that the period during which serum can stimulate the bacteria to produce coagulase corresponds to the lag-phase of growth.

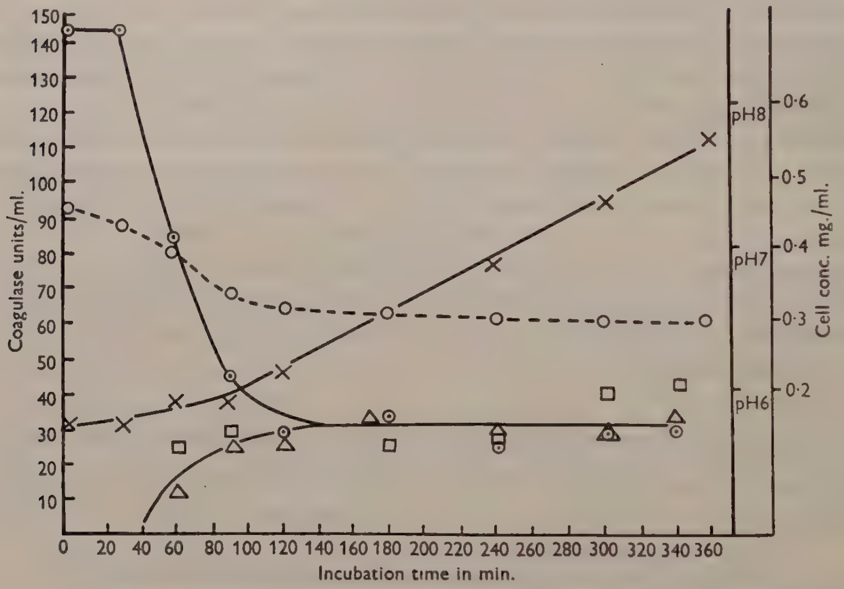


Fig. 1. Influence of rabbit serum on the production of coagulase by staphylococci.

Organism incubated for varying periods in brain-heart infusion; ⊙ — ⊙, rabbit serum added and mixture incubated 30 min. and then centrifuged; □ — □, culture centrifuged and supernatant incubated 30 min.; △—△, culture centrifuged, rabbit serum added to supernatant and mixture incubated for 30 min.; × — ×, concentration of bacteria as determined by opacity tubes; ○ - - - ○, pH of culture in brain-heart infusion.

Rabbit serum exerts its maximal effect with great rapidity provided that the bacteria are at the appropriate stage of growth (i.e. towards the end of the lag-phase; Table 3).

Table 2. *Influence of rabbit serum on the production of coagulase by staphylococci; effect of adding serum to cells of varying age*

Strain: E.B.1.; 0·4 mg. dry wt. cells/ml. Medium: brain-heart infusion, pH 7·6. Incubation temperature: 37°.

Age of cells (min.)	Conc. of cells (mg./ml.)	Supernatant from serum with cells for 30 min. (C)		Serum with supernatant from cells for 30 min. (B)		Supernatant from cells (A)	
		pH	Coagulase units/ml.	pH	Coagulase units/ml.	pH	Coagulase units/ml.
0	0·16	7·23	143	7·21	<10	7·37	<10
30	0·16	7·07	143	7·11	<10	7·26	<10
60	0·19	6·78	84	6·84	12	7·07	25
90	0·19	6·63	45	6·65	25	6·83	29
120	0·23	6·60	29	6·52	25	6·73	29
180	0·32	6·57	33	6·46	33	6·66	25
240	0·38	6·55	25	6·43	29	6·63	27
300	0·42	6·63	29	6·42	29	6·64	40
360	0·56	6·50	29	6·42	33	6·64	45

See Fig. 1.

Table 3. *Influence of rabbit serum on the production of coagulase by staphylococci: effect of varying the age of cells and the time of their contact with serum*

Strain: E.B.1; 0·3 mg. dry wt. cells/ml. Medium: brain-heart infusion + 10 % rabbit serum, pH 7·3. Incubation temperature, 37°.

Period of incubation of cells in broth before addition of serum (min.)	Coagulase units/ml. formed after serum has been in contact for				
	5 min.	10 min.	20 min.	30 min.	60 min.
0	<10	<10	<10	15	30
10	<10	<10	<10	15	15
20	15	15	15	20	40
30	40	60	50	50	50
40	60	60	50	60	100
60	100	100	100	80	80

Horse serum, horse plasma and sheep serum are less effective than rabbit serum. With each of these the highest titre of coagulase is reached at about the same time as with rabbit serum and further incubation does not cause the titre to decline. Egg yolk is about as active as horse serum. Egg white, ribo- and deoxyribonucleic acids are inactive. Rabbit serum when treated with pepsin loses its power to promote the formation of coagulase, indicating that the active portion is either a protein or is associated with a protein. Increase in the concentration of rabbit serum above 10 % does not result in increased production of coagulase; the optimal concentration is in the neighbourhood of 10 %. A large excess (80 %) of serum decreases the yield of coagulase, probably because of the presence of an inhibitor in the serum.

Experiments on the broth-factors necessary for coagulase production

All the foregoing experiments were done using Difco brain-heart infusion as the source of 'broth-factors'. Our efforts were then directed to find a medium simpler than this complex infusion. We found that a medium containing peptone, sodium chloride, glucose and phosphate buffer at pH 8·0 could partially replace the infusion, as shown in Table 4. In these and in all subsequent experiments in this section, 10 % rabbit serum was used as source of serum-factor. The amount of coagulase produced was largely independent of the concentration of peptone between 0·5 and 5·0 % and of the concentration of glucose, also between 0·5 and 5·0 %. The ratio of activity between brain-heart infusion and the optimal glucose-peptone medium varied slightly in different experiments but on the whole the infusion was rather more efficient.

Table 4. *Effect of varying the concentration of glucose and peptone on the production of coagulase by staphylococci*

Strain: B.B.; 0·35 mg. dry wt. cells/ml. Medium: Evans Peptone; 0·5 % glucose; NaCl; 0·25 % Na_2HPO_4; 10 % rabbit serum; pH 7·9. Incubation temperature 37°.

Peptone (%)	Glucose (%)	Coagulase units/ml. formed in 1 hr.
5·0	0·5	130
2·0	0·5	170
1·0	0·5	140
0·5	0·5	130
1·0	0	70
1·0	1·0	140
1·0	2·0	160
1·0	5·0	130
Brain-heart infusion, pH 7·3:		140

Ether-extraction of the brain-heart infusion did not alter its capacity to serve as a medium for coagulase production and addition of the extracted lipids to the peptone-glucose solution did not improve the latter. Acid-hydrolysed casein was about half as efficient as peptone when both were prepared in 0·5 % glucose buffer. The yield of coagulase was independent of the concentration of casein hydrolysate between 0·5 and 5·0 %. We considered it possible that peptone and casein hydrolysate were less efficient than the infusion because they were poorer as growth media, i.e. they would prolong the lag-phase so that the period of maximal coagulase production would be reached later. However with three concentrations of peptone the yield of coagulase after incubation for 2 hr. was the same as that after 1 hr.

Influence of pH on the production of coagulase

It can be seen from Table 2 that, concurrent with the production of coagulase in the complete medium, there is a rapid fall in the pH value of the medium. When the pH of the medium was kept between 7·3 and 7·6, the maximum yield of coagulase was reached rather earlier but was not higher than that produced when the pH value was allowed to fall (Table 5). In these experiments, again, the results indicated that coagulase was produced during the lag-phase of growth.

Table 5. *Influence of pH value on production of coagulase by staphylococci; comparison of neutralized with non-neutralized medium*

Strain: B.B.; 0·47 mg. dry wt. cells/ml. Medium: brain-heart infusion+10% rabbit serum. Incubation temperature 37°.

	pH not controlled			pH kept at between 7·3–7·6		
Period of incubation (min.)	pH	Cell conc. (mg./ml.)	Coagulase units/ml.	pH	Cell conc. (mg./ml.)	Coagulase units/ml.
0	7·30	0·47	0	7·30	0·47	0
10	7·20	0·47	35	7·30	0·47	<10
20	7·16	0·47	120	7·38	0·47	10
30	6·99	0·59	170	7·40	0·47	145
40	6·89	0·71	120	7·34	0·59	200
50	6·89	0·71	170	7·60	0·59	250
60	6·73	0·75	200	7·60	0·85	200
90	6·53	0·85	290	7·45	0·85	200
120	6·47	0·94	290	7·34	0·85	200
180	6·42	0·94	290	7·30	0·85	200
360	6·34	0·94	200	—	—	—

The yield of coagulase does, however, depend upon the initial pH value of the medium as is shown in Table 6 and Fig. 2. With an initial pH of 6·1 no coagulase was produced. Increase in initial pH resulted in increased yield of coagulase which reached a maximum with initial pH values between 8·4 and 9·3. With an initial pH value of 9·3 the rate of coagulase production was slower but the final yield was greater (Fig. 2). Fig. 3 shows that coagulase is produced when the actual pH of the medium is between about 7 and 8·5 with a maximum yield at pH 7·6.

Influence of aeration on the production of coagulase

When the medium was gently stirred, by directing a stream of air on to the surface, more coagulase (660 units/ml.) was produced than in a control non-aerated flask (330 units/ml.). Incubation beneath a vaseline seal resulted in a lower yield of coagulase (100 units/ml.). No attempt was made to separate the effects of aeration and agitation.

Table 6. *Influence of the initial pH of the medium on the production of coagulase by staphylococci*

Strain: E.E.; 0·4 mg. dry wt. cells/ml. Medium: 1% proteose peptone; 0·5% NaCl; 0·25% Na_2HPO_4; 10% rabbit serum. Incubation temperature 37°.

Period of incubation							
0 min.		30 min.		60 min.		90 min.	
pH	units/ml.	pH	units/ml.	pH	units/ml.	pH	units/ml.
6·15	<10	5·85	<10	5·62	<10	5·56	<10
6·92	<10	6·67	29	6·50	33	6·46	40
7·62	<10	7·21	67	6·95	84	6·89	84
8·39	<10	7·66	84	7·26	84	7·24	100
9·30	<10	8·40	29	7·67	84	7·61	120
9·95	<10	10·03	<10	9·93	<10	9·74	<10

See Fig. 2.

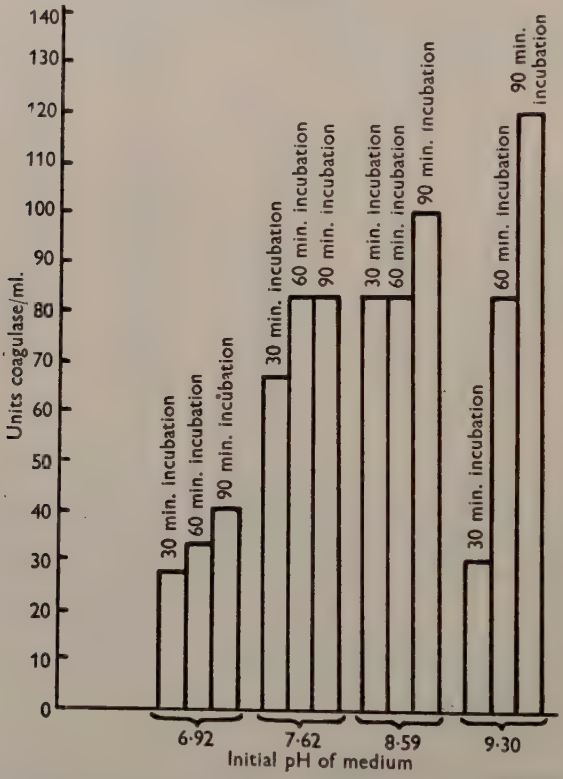


Fig. 2. Influence of the initial pH of the medium on the production of coagulase by staphylococci in glucose-peptone medium containing rabbit serum.

Influence of cell concentration

For each strain of organism there is an optimum concentration of staphylococci for the production of coagulase. For instance, *Staph. aureus* strain BB in brain-heart infusion with 10 % rabbit serum produced 200 coagulase units/ml. when the cell-concentration was between 3·5 and 7·0 mg. dry wt./ml.; at 35 mg./ml. only 50 units coagulase/ml. were produced.

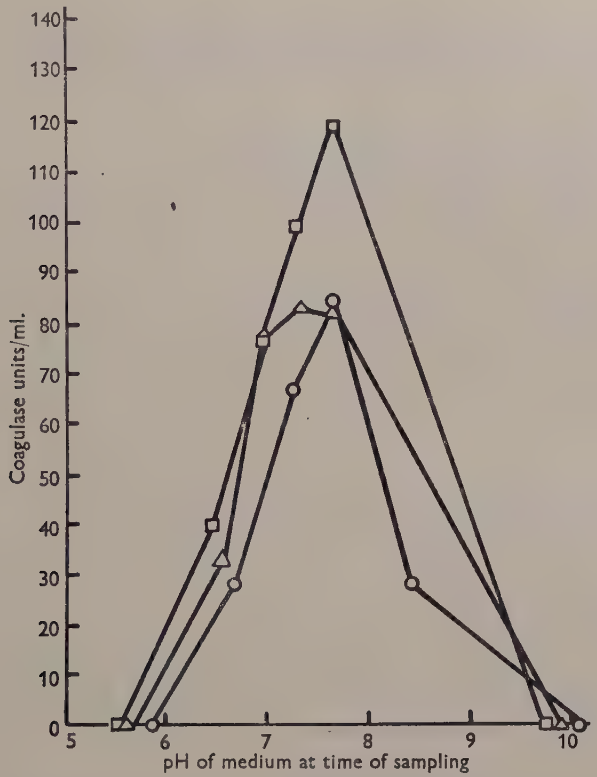


Fig. 3. Relationship between actual pH of medium at time of sampling and the concentration of coagulase contained in the sample. Results from experiment which is also illustrated in Fig. 2. ○ — ○, 30 min. incubation; △ — △, 60 min. incubation; □ — □, 90 min. incubation.

Influence of temperature

Coagulase is produced at temperatures between 20 and 40°, with a maximum yield at about 35°. An experiment in which the staphylococci were incubated in brain-heart infusion with 10 % rabbit serum at various temperatures gave the following results: 2°, <5 units coagulase/ml.; 20°, 40 units/ml.; 30°, 200 units/ml.; 35°, 250 units/ml; 40°, 140 units/ml.; 45° <5 units/ml.

Influence of concentration of salt

The total salt concentration influences the yield of coagulase. An optimal salt concentration was reached at 0·5 % NaCl with 0·25 % Na_2HPO_4. With concentrations of NaCl greater than 5 % no coagulase was produced.

DISCUSSION

At a particular phase of their growth, corresponding to the latter part of the lag-phase, staphylococci, in defined conditions of cell-concentration, pH, temperature and composition of medium, and in the presence of a protein in rabbit serum, produced a large amount of coagulase within a few minutes. In the absence of serum under the same conditions only a small amount of pro-coagulase was produced. These observations may be interpreted in two ways: (1) The cells form pro-coagulase in large amounts on their surface during the lag-phase. Normally this pro-coagulase is mainly bound to the surface and only a small proportion is shed into the medium. Serum-factor disrupts this bond with the consequent liberation of pro-coagulase and the formation of coagulase. As the age of the cells increases, during the phase of active cell-division, the pro-coagulase at the cell-surface, but not that in the surrounding medium, is destroyed and the subsequent addition of serum is without effect. (2) The cells form pro-coagulase in small amounts during the lag-phase and shed it in the surrounding medium. Serum-factor accelerates this formation of pro-coagulase. When cell-division starts, pro-coagulase is no longer produced.

We have seen from the experiment shown in Table 3 that serum exerts its effect with great rapidity since, when the cells are 60 min. old, serum can cause the formation of a maximal amount of coagulase in less than 5 min. The speed of this reaction is more in accordance with the hypothesis that already-formed pro-coagulase is liberated, than that which assumes the production of fresh coagulase. This transient production of a substance by young cells is reminiscent of capsule formation (Lyons, 1937). The possible identity of the serum-factor with the reaction-factor of Smith & Hale (1944) remains to be considered. The broth-factors appear to function simply as nutrients, i.e. they assist the cells to start their growth-cycle.

If these results can be applied to the more complex conditions obtaining *in vivo*, they enable us to put forward the following picture of the sequence of events taking place in the formation of a staphylococcal lesion. When bacteria gain access to the tissues, there is a time-lag before cell-division starts. During this lag-period the defence mechanisms of the host are being mobilized with great speed and the organism must protect itself with even greater speed if it is to establish itself. We have seen from the experiments described above that staphylococci can produce coagulase within a few minutes. Hale & Smith (1945) have shown that coagulase forms a barrier of fibrin round the bacteria and protects them from phagocytosis. The production of too much coagulase would be a liability to the organism, since an impermeable barrier of fibrin would prevent diffusion of nutrient material, and would also be a mechanical hindrance to colonial expansion. But we have shown that coagulase is produced only during the lag-phase, and furthermore, certain strains of staphylococci can produce a fibrinolysin (Christie & Wilson, 1941) which will destroy the already-formed barrier. According to this view, therefore, the establishment of a staphylococcal lesion is an orderly sequence of events: the initial inoculum is protected from phagocytosis by a barrier of fibrin; later this barrier is removed and by this time the cells have elaborated a series of toxic principles (haemolysins, leucocidins, hyaluronidase, etc.) to assist them in their conflict with the phagocytes and to further the development of the lesion.

I wish to record my gratitude to Dr J. M. Pryce for his help with various chemical aspects of this work, particularly with regard to the establishment of the assay process.

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The Determination of Small Quantities of Bacteria by means of the Biuret Reaction

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SUMMARY: The serum-protein determination method of Robinson & Hogden (1940), using the biuret reaction, has been applied to the determination of small quantities of bacteria. This 'direct protein' estimation involves about three-quarters of the total protein of the bacteria.

When a suspension of coliform bacteria, suitably washed and suspended in distilled water or saline, is treated with sodium hydroxide and copper sulphate, according to the method of Robinson & Hogden (1940) for the determination of serum proteins, and the excess of copper hydroxide removed by centrifugation, a clear purple solution is obtained. The intensity of the colour is linearly proportional to the quantity of bacteria present over a wide range, and its measurement in the Spekker photoelectric absorptiometer provides a rapid and accurate means of determination. The method has proved most useful for small quantities of bacteria, as a micro-method using the 0·5 ml. cells of the absorptiometer. When enough material is available, the larger (10 ml.) cells of the absorptiometer may be used with some gain in accuracy.

Some other groups of bacteria, and yeasts, do not react in this way, but give a biuret colour which is chiefly bound to the cells. In these cases (e.g. *Staphylococcus aureus*; 'baker's yeast') the biuret colour may be obtained in soluble form if the mixture of bacteria and sodium hydroxide be heated in a boiling water-bath for a few minutes and then cooled, before the addition of the copper sulphate.

EXPERIMENTAL

Preparation of suspensions. The suspensions were prepared without special treatment for use in various enzyme studies. Liquid cultures were centrifuged, the bacteria suspended in distilled water and filtered through glass wool, recentrifuged and finally resuspended in distilled water. From solid media the bacteria were removed by gentle shaking with distilled water, the suspension filtered through glass wool, centrifuged and the bacteria washed once and again resuspended in distilled water.

Calibration of the absorptiometer scale

A series of volumes of a bacterial suspension containing up to 5 mg. dry weight of bacteria was measured out into small test-tubes, water added to make each sample up to 1·65 ml., and each treated with 0·3 ml. 20 % (w/v) NaOH, and then 0·05 ml. 25 % (w/v) $CuSO_4.5H_2O$ solution. The copper hydroxide precipitate so obtained was broken up with a glass rod, and the tubes centrifuged at 3,500 r.p.m. for 15 min. The supernatant solutions were decanted and their colour intensities read on the Spekker absorptiometer, using the 0·5 ml. micro-cells, and the yellow-green filter.

On the same suspensions determinations of total N (by micro-Kjeldahl) or dry weight or both were carried out. The Spekker readings, plotted against total N or dry weight, gave a straight line (Fig. 1). This calibration has been done with a number of strains of *Bacterium coli*, with reasonably concordant results (see below). A strain of *Aerobacter aerogenes* gave results similar to those obtained with *Bact. coli* (see Table 2).

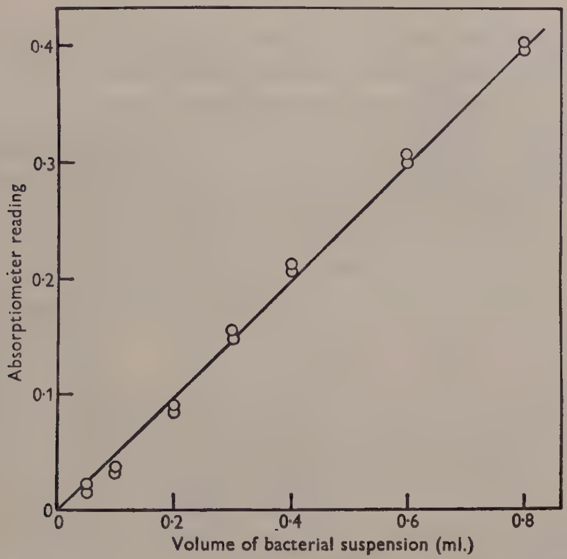

Fig. 1. The relationship between quantity of bacteria and absorptiometer reading. The suspension used contained 6·25 mg. dry wt./ml. (=8·13 mg. 'protein'/ml.).

Calibration in terms of bacterial protein

It is of interest to know what proportion of the total protein of bacteria is involved in giving this direct biuret reaction. In order to translate the colour readings into terms of absolute quantities of protein, it was necessary to measure the biuret colour given by bacterial protein, and use this as a standard. Samples of bacterial protein were obtained by treating cell suspensions with NaOH to a final concentration of 3 %, spinning out the cell residues, and precipitating the protein by the addition of 25 % trichloracetic acid solution to give a final concentration of 5 % of the acid. The precipitation of the opalescent colloidal solution was completed by the addition of ethanol to about 33 % by volume. The protein was washed once with 5 % trichloracetic acid, dissolved in water with the help of a minimal amount of NaOH, and filtered. This solution was used for biuret colour measurements and total N determinations (Table 1).

Table 1. *Comparison of intensity of biuret colour obtained with rabbit-serum protein and with various samples of protein from* Bact. coli

Protein preparation	Absorptiometer reading for 1 mg. protein
Rabbit serum	0·152
Bact. coli (mixed strains)	0·167
(strain A)	0·150
(strain B)	0·156
(strain A)	0·161
(strain AD)	0·167
Average for *Bact. coli*	0·160

Table 2. *The relation of dry weight, total N, and protein content as determined by the biuret reaction, in suspensions of* Bact. coli *and* A. aerogenes *in distilled water*

Species and strain	Conditions of growth	Duration of growth (hr.)	Dry wt. mg./ml.	Total N mg./ml.	'Protein' mg./ml.	TotalN / Dry wt. %	'Protein' / Dry wt. %	'Protein'N / Total N %
Bact. coli								
B	Broth agar, 37°	16–20	7·7	0·95	3·67	12·4	48	64
C	Broth agar, 37°	16–20	8·1	1·01	3·91	12·4	49	64
X	Broth 37°	16–20	11·75	1·38	5·7	11·8	49	70
1	— —	18	3·4	0·41	1·74	12·0	51	71
4	— —	18	5·5	0·85	3·40	15·4	62	66
14	— —	20	5·3	0·69	2·54	13·0	48	61
AD	— —	20	5·9	0·77	2·87	13·1	49	62
1R	— —	18	—	0·099	0·44	—	—	74
4R	— —	18	—	0·120	0·46	—	—	64
1R	Glucose broth 37°	15	—	0·246	0·89	—	—	60
4R	Glucose broth 37°	15	—	0·180	0·64	—	—	59
1	Glucose broth 37°	15	2·45	0·315	1·22	12·8	50	65
4	Glucose broth 37°	15	2·35	0·305	1·12	13·0	48	61
1	Glucose broth 37°	15	7·2	0·84	3·15	11·7	44	62
1R	Glucose broth 37°	15	4·4	0·62	2·28	14·0	52	61
1	Broth 37°	16–20	6·6	0·74	3·04	11·2	46	69
A. aerogenes	Glucose broth 30°	8	4·64	—	2·25	—	48	—
	30°	16	3·38	—	1·70	—	50	—
	30°	24	4·25	—	2·08	—	48	—
	30°	16	2·72	—	1·42	—	52	—
	37°	16	3·44	—	1·73	—	50	—
	26°	16	1·06	—	0·59	—	55	—
	30°	16	1·18	—	0·58	—	50	—
	34°	16	1·57	—	0·72	—	46	—
	Averages; (a) *Bact. coli*					12·7	50±4	65±4
	(b) *A. aerogenes*					—	50±2	—

* 'Protein' refers to 'directly estimated protein'. 'Protein N' is derived from 'Protein' by dividing the latter by 6·0.

The relationship between absorptiometer reading and absolute amount of bacterial protein having been established in this way—the ratio of 0·160 did not differ much from that for serum protein (0·152)—it was now possible to determine the value of the ratio 'protein directly estimated by the biuret reaction': 'total N'. Various suspensions of cells, from a number of strains of *Bact. coli* and a single strain of *A. aerogenes*, grown under different conditions, were submitted to estimations of (*a*) dry weight, (*b*) total N by Kjeldahl, and (*c*) 'directly estimated protein' by the biuret reaction. In Table 2 it will be seen that variations of strain and growth conditions did not lead to very marked differences in the ratios of these quantities.

Application to other micro-organisms

When the above method was applied to suspensions of yeast the final solution obtained was almost colourless and the centrifuged deposit of cells and copper hydroxide had a purple tinge. However, if after the addition of the sodium hydroxide the mixture were heated in a boiling water-bath for periods up to 10 min., and then cooled before the addition of the copper sulphate solution, the results in Table 3 were obtained. The 'protein' determination after some 5 min. reached a maximum, which was reasonably stable to further heating.

Table 3. *The effect of heating for various times in a water-bath at* 100° *on the intensity of the biuret colour produced by micro-organisms*

	Time of heating (min.)				
	0	1	2	5	10
	Absorptiometer reading				
Organism					
Staph. aureus	0·095	0·285	0·413	0·435	0·412
'Baker's yeast'	0·065	0·491	0·533	0·545	0·531
Bact. coli (strain 7)	0·468		0·481	0·469	0·458
Bact. coli (strain 8)	0·480		0·489	0·490	0·475

Certain bacteria behaved in the same way (e.g. *Staph. aureus*, see Table 3) and although this matter has not been explored any further, it seems likely that the method would prove to be of general applicability, provided that the step of heating for 5 min. at 100° were included. Table 3 also illustrates the point that with coliform bacteria heating in this way brings about no significant increase in the yield of protein.

With *Staph. aureus*, a single determination on each of five strains gave figures for the ratio 'protein': dry weight of 46, 48, 46, 50, and 45 % (average 47 %). A single determination on a sample of 'baker's yeast' gave a value of 71 %. Clearly with other micro-organisms the value of this ratio would have to be determined afresh.

DISCUSSION

Tables 1 and 2 show that the greater part of the protein of bacteria will react with alkaline copper solution to give a biuret colour which is soluble in water and can serve as a means of measurement of the quantity of bacteria present. In various strains of *Bact. coli* the ratio 'directly estimated protein' N : total N is fairly constant, with an average value of 65 %. Just how much of the total N of bacteria is non-protein N is uncertain; Boivin & Mesrobeanu (1934; quoted in *Topley & Wilson's Principles of Bacteriology and Immunity*, 1946) say that not less than 80 % of the total bacterial N is protein N, but the information is scanty and unreliable. If 85 % be adopted arbitrarily as a probable figure for the ratio protein N : total N, then the ratio 'directly estimated protein': total protein has an average value of 76 % (and it clearly could not in any case be less than 65 %). It is rather surprising to find that so large a proportion of the total protein of bacteria will react directly with copper in alkaline solution in this way, but the fact may give greater confidence in the use of the method for the determination of quantities of bacteria.

If a comparison of this method with those previously available is made, the following points may be noted. Total N by micro-Kjeldahl, though more sensitive and showing slightly greater accuracy, requires more care and much more time. Direct dry weight measurements, to give the same degree of accuracy, require a much greater amount of material. Turbidimetric methods, though simple, are less accurate.

The optimal quantity of bacteria for a micro-determination is about 2·5 mg. of protein (0·5 mg. N or 5 mg. dry wt.) with a probable error of about ±2 %, and amounts down to 0·5 mg. of protein or less may be determined with confidence though with a corresponding decrease in accuracy. Under the conditions described, the quantity used for a determination must not exceed about 3 mg. of protein, as above that limit the intensity of the colour is no longer proportional to the quantity of bacteria. These estimates of accuracy apply, of course, only to the figures for 'directly estimated protein'; if they have to be converted to 'total N' or 'dry weight', slightly greater uncertainty is introduced, as can be seen from Table 2. For many purposes, however, the protein content of a culture or suspension of bacteria might be more relevant than either its dry weight or its N content.

The method might prove useful for comparing amounts of growth on different media or under different conditions. In this case the bacteria would have to be freed by washing from any protein components of the medium, and also from substances such as ammonium salts and amino-acids which give a blue colour with the copper reagent. Alternatively, interference from these latter substances could be eliminated by using a blue filter in the place of the yellow-green in the absorptiometer, but with loss of about one half of the sensitivity.

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Osmophilic Yeasts in Raw Beet and Cane Sugars and Intermediate Sugar-Refining Products

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SUMMARY: Osmophilic yeasts are found in the film of molasses on the crystals of raw sugars and in intermediate sugar-refining products of a wide range of concentration. They are allied to yeasts found in similar situations such as concentrated fruit juices, etc., and can be made to spore more or less freely under dry conditions, usually with conjugation. They are unable to destroy sucrose in strongly buffered solutions; some are able to produce sufficient acid from traces of invert sugar present gradually to invert the sucrose. During this process multiplication takes place. Previous workers have shown that the majority of organisms of this group is highly acid resistant.

Organisms which grow in a wide range of concentration of dissolved solids (salts, sugar) show, at acid pH values, an adaptation to growth in higher concentrations accompanied by a diminished metabolic rate. This is lost after a period of 6–8 weeks in dilute media, but can be revived in many cases by increasing the sugar concentration in not less than two steps. Temperature resistance is also increased in concentrated media.

According to Henrici's monograph (1941) the term osmophilic was first applied by Richter in 1912 to micro-organisms which multiply in solutions of high osmotic pressure. Apart from the brine yeasts, this term is most frequently applied to the yeasts tolerant of high sugar concentration to be found in a wide range of natural and manufactured products. Nussbaumer (1910) was the first to find that osmophilic yeasts were a factor in honey spoilage, and Lochhead & Heron (1929) traced these to the honey of various flowers. Kroemer & Krumbholtz (1931) studied similar organisms in fermenting wine musts produced from grapes infected with *Botrytis cinerea*; Baker & Mrak (1938) found organisms from the same group on crystallized fruit; Sacchetti (1932, 1939) studied the microflora of fruit juices, Italian wine musts and honey; Owen (1948) has reported the presence of *Torulopsis* spp. in sugars and molasses.

More detailed diagnostic work on honey by Lochhead & Farrell (1931) and Lochhead & McMasters (1931), on honey and wine musts by Sacchetti (1932, 1939), and on fruit juices by Ingram (1949 *a*, *b*) showed that the majority of organisms involved belonged to the sub-genus *Zygosaccharomyces* Barker and many were osmophilic strains of known species. Kroemer & Krumbholtz (1931) divide the osmophilic zygosaccharomyces on physiological grounds into two groups:

(1) Those which will only grow in solutions of high concentration are able to ferment disaccharides and produce little volatile acid.

(2) Those which will grow in solutions of a wide range of concentration, so that they tolerate rather than require concentrated solutions; they ferment much less vigorously than Group 1, and produce varying quantities of volatile acid.

The present investigation was concerned with the isolation of osmophilic yeasts from raw sugar and intermediate refinery products, with a view to physiological and ecological study.

PRELIMINARY STUDY

Yeasts in sugar can be traced back to the original cane (Pl. 1, figs. 1 and 2); in raw sugar they are found in the film of molasses round each crystal, and when crystals are mounted in a little water, these yeasts can be seen under the microscope streaming away from the surface of the crystal as the molasses mix with the surrounding medium. Hyphae of the *Aspergillus glaucus* group of moulds are occasionally found there (Pl. 1, fig. 3), but the yeasts are far more common, and, in a water-damaged sugar, may be as high as 5 million/g. sugar (de Whalley & Scarr, 1947).

So far, forty strains of yeast have been isolated from raw cane sugars from the West Indies, Africa and Australia, as well as from a few samples of beet grown in Gt. Britain, and from intermediate refinery products. Most of these yeasts form asci under standard conditions (Phaff & Mrak, 1948, 1949) provided the medium be dry, although usually only sparsely (Pl. 1, fig. 4). Asci are formed with or without previous anisogamous conjugation, and 1 to 4 (usually 2) ascospores are produced. A sediment is produced in wort and in some cases also a surface ring. Pseudomycelium when formed is normally in the sediment, so that lack of oxygen may stimulate this formation (Pl. 1, fig. 5).

All forty yeasts appear to belong to Kroemer & Krumbholtz's second group, as they are tolerant of a wide range of sugar concentrations similar to those found in fruit juices (Ingram, 1949 *a*, *b*). Similarly, these yeasts gradually lose this property when kept in dilute solutions for 6 to 8 weeks. It may, however, be quickly regained by increasing the concentration in at least two steps. In spite of the large excess of sucrose present, under natural conditions, they show the property of this group that they are unable to ferment sucrose, confirming earlier reports from Browne (1908) on the microflora of sugars. However, previous observations in these laboratories on the destruction of sugar in unsterilized refinery syrups showed large numbers of these organisms which multiplied slowly, even in the refrigerator. Therefore one of the most active yeasts was grown in a medium containing 40 % sucrose in aerated culture, and then centrifuged and allowed to autolyse under toluene. Neither the yeast nor the autolysate caused any inversion when left with a fresh 5 % sucrose solution overnight at room temperature, so that it was concluded that the yeast contained negligible quantities of sucrase (Kluyver & Custers, 1940). The standard plate auxanogram described by Wickerham & Burton (1948) similarly gave no results, but with their liquid auxanogram, some yeasts did show positive results after 2 to 3 weeks, provided yeast extract were present, thus indicating that sucrose destruction does take place, either through an adaptive enzyme or by some other means.

The main part of this work was planned on the above findings to show what the nature of high sugar-tolerance might be, and how sucrose destruction might take place.

METHODS

The greater part of the work was carried out with a strain of *Zygosaccharomyces* sp., strain 21, isolated from a Cuban raw sugar; this was a single-cell isolate, selected because it gave a positive auxanogram for sucrose. For comparative purposes, strain 32, a single-cell isolate from a Natal raw sugar, which gave a negative auxanogram with sucrose, and a culture of *Saccharomyces carlsbergensis* Hansen from the Centraal Bureau voor Schimmelcultures, Delft, Holland, were used. The osmophilic yeasts were stored on a special medium used in these laboratories (de Whalley & Scarr, 1947) consisting of an artificial wort agar made up in a 45 % solids syrup containing glucose and sucrose. The *S. carlsbergensis* was stored on Difco wort agar.

The osmophilic yeasts were 72-hr. cultures, and the other yeasts 48-hr. cultures, because of their faster rate of multiplication. The yeast extract used was Yeastrel (Brewers' Food Supply Co. Ltd.). Cultures for inoculation were suspended in a small portion of the reaction medium immediately before use. Citric acid-disodium phosphate buffer (McIlvaine; see Britton, 1944) was used throughout as the yeasts gave a negative auxanogram with citric acid and appeared unaffected by excess phosphate. All experiments were made in unaerated solutions unless otherwise stated, using 20 ml. of medium in 6 in. × 1 in. test-tubes.

All sugar solutions were sterilized by filtration. The following media were used: (1) for dilute solutions in the physical investigations: sucrose 5 %; glucose 1 %; Evans Bact. Peptone 0·5 %; KH_2PO_4 (Analar) 0·1 %; $MgSO_4$ (Analar) trace; $FeSO_4$ (Analar) trace; (2) for the concentrated solutions: 54 % w/w of sucrose instead of 5 %; (3) for the nutritional investigations: sucrose 50 % w/w; glucose 1 % w/w; KH_2PO_4 (Analar) 0·1 %; $(NH_4)_2SO_4$ (Analar) 0·5 %; $MgSO_4$ (Analar) trace; $FeSO_4$ (Analar) trace.

RESULTS

Visual observations

Old cultures had a strong surface ring in concentrated media with both osmophilic yeasts, but only strain 21 formed one in dilute media. With both strains under anaerobic conditions in concentrated solution the yeasts grew in clumps which appeared in nigrosine to have capsules; these might have been merely layers of concentrated syrup, as they gradually diffused into the surrounding medium. In aerated solutions, cells of strain 21 were seen singly or in pairs, and the apparent capsule was still present.

On transference to high concentrations of sucrose, 50–90 % of the yeast cells assumed a transient shrunken condition with dense cell contents and became half their normal size. This appeared to be some form of plasmolysis, as the cells recovered under conditions where multiplication could take place. Normal plasmolysis was seen occasionally. There was little change in the size of actively growing cells at different concentrations, although they were slightly smaller in the stronger solution.

The relationship between temperature and the concentration of solids in the medium

Media 1 and 2 were buffered with 0·1 M citric acid and 0·2 M sodium phosphate to pH 4·5, inoculated with suspensions of cultures of strain 21, strain 32 or *S. carlsbergensis*, and incubated at 25°, 35°, and 41°, and, for the higher concentration of sucrose, at 50°. Growth at 4° was omitted as being too slow.

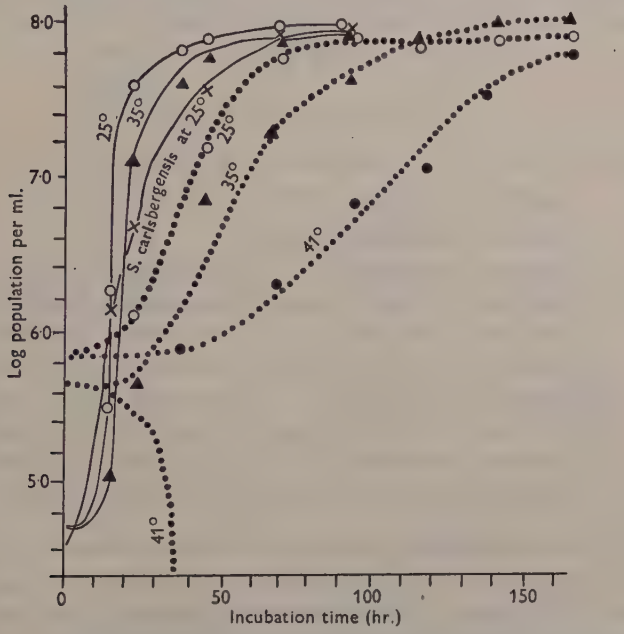

Fig. 1. Variation in growth of strain 21. *Zygosaccharomyces* sp. with concentration and temperature. ••••• , growth in solutions containing 54 % w/w sucrose; ——, growth in solutions containing 5 % w/w sucrose.

Growth was followed by haemacytometer counts (Fig. 1). The effect of increased concentration is in each case to decrease the multiplication rate, and to raise the optimum temperature from 25° to 35°.

These results are quite consistent if it be assumed that the cells adopt a latent state somewhere between that of actively dividing cells and such a latent form as a bacterial spore. As absorption both of water (Bennet-Clarke, 1948) and nutrients (Hoagland, 1944) depends directly on metabolic activity in all plant cells, it is reasonable to suppose that, as metabolism continues at a much slower rate under physiologically drier conditions autolysis is delayed and the transient shrunken condition of the cells is overcome. If this is so, it explains why the optimum temperature is higher under conditions of higher concentration; on the other hand, it is surprising that the decrease in multiplication rate is not even greater.

The relationship between pH value and the concentration of solids in the medium

Media 1 and 2 were buffered with 0·1 M citric acid and 0·2 M sodium phosphate to pH values between 2·5 and 7·5 and inoculated with strain 21. All cultures were incubated at 25°. Growth was followed by haemacytometer counts (Fig. 2).

It appears that for strain 21 the pH value is a controlling factor in the adaptation to growth in solutions containing higher concentrations of sucrose. These results also demonstrate the acid-resistance of the yeast used and its similarity to other yeasts (Nickerson, 1943) of this group.

Nutritional requirements

Medium 3 was used in ten media made up as follows:

i. Medium 3 complete.
ii. Medium 3 without glucose.
iii. Medium 3 complete + isoleucine 10 μg./ml.
iv. Medium 3 without glucose + isoleucine 10 μg./ml.
v. Medium 3 complete + glutamine 10 μg./ml.
vi. Medium 3 without glucose + glutamine 10 μg./ml.
vii. Medium 3 complete + aspartic acid 10 μg./ml.
viii. Medium 3 without glucose + aspartic acid 10 μg./ml.
ix. Medium 3 complete + Yeastrel 10 μg./ml.
x. Medium 3 without glucose + Yeastrel 10 μg./ml.

The media were inoculated with a suspension of strain 21, and duplicates of media i, ii, ix and x were inoculated with a suspension of strain 32. Growth was followed as before. All solutions were buffered to pH 4·5 with 0·01 M sodium phosphate and 0·01 M citric acid and incubated at 25°.

Tubes of media i, ii, ix and x were first inoculated with strain 21 or strain 32 (Fig. 3). There was a long lag period before multiplication began in the medium lacking glucose, and during that period, 99 % of the cells were in the shrunken condition. The effect of adding yeast extract to the medium not containing glucose was at first sight peculiar with strain 21. Some residual nutrients were introduced with the inoculum, which caused slight multiplication of the more active yeast. During the ensuing stationary phase, the pH values dropped from 4·6 to 3 and multiplication began again. Equivalent amounts of amino-acids produced the same effect with strain 21, as shown in Fig. 4.

Cultures left for more than ten days continued to multiply at a much slower rate. Counts after three weeks of cultures of strain 21 containing no

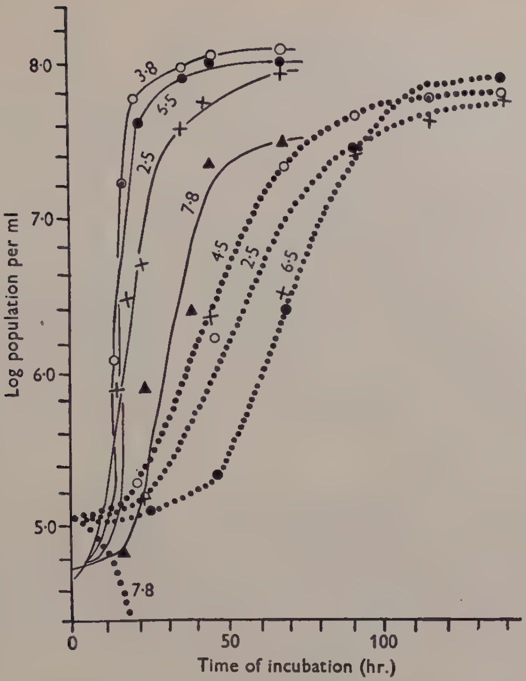

Fig. 2. Variation in growth of strain 21. *Zygosaccharomyces* sp. with concentration and pH. ••••••••, growth in solution containing 54 % w/w sucrose; ——, growth in solution containing 5 % w/w sucrose.

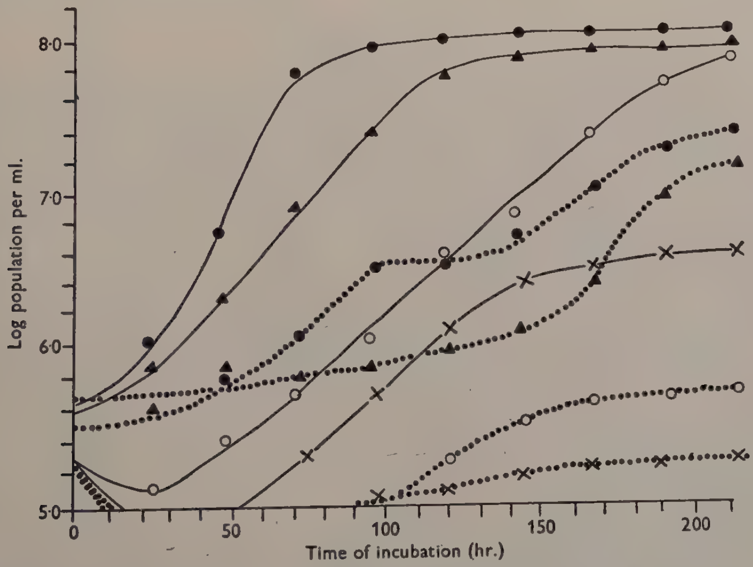

Fig. 3. Effect of Glucose (1 %) and Yeastrel (10 μg./ml.) on growth of strains 21 and 32. *Zygosaccharomyces* spp. ——, medium containing 50 % w/w sucrose and 1 % w/w glucose; ••••••••••••••, medium containing 50 % w/w sucrose but no glucose; —●—●—, strain 21 with 10 μg. added Yeastrel/ml.; —▲—▲—, strain 21 without 10 μg. added Yeastrel/ml.; —○—○—, strain 32 with 10 μg. added Yeastrel/ml.; —×—×—, strain 32 without added Yeastrel.

original glucose were: Blank, complete medium without glucose=$9{,}800\times10^4$ cells/ml.; the same, plus yeast extract=$20{,}500\times10^4$ cells/ml.; the same, plus isoleucine=$19{,}600\times10^4$ cells/ml.

In every case, the final pH value for strain 21 was approximately 2·8, and for strain 32, 3·2 after nine days. There was little further drop at the end of three weeks when the pH was approximately 2·4. In order to trace this fall in pH in more detail, the complete medium was made up buffered with 0·01 M citric acid and 0·02 M sodium phosphate to pH 4·7, and containing a small quantity of initial dextrose to accelerate the reaction; 400 ml. were incubated at room temperature, subjected to gentle aeration (by a 1/150 h.p. rotary pump (Charles Austin & Co.) mainly to keep the solution stirred and to avoid the accumulation of any carbon dioxide. The culture was examined at intervals for cell content, pH (glass electrode), refractrometric solids, and at the start and finish for invert by the methylene blue method (de Whalley, 1937) (Fig. 5).

Here there was sufficient acid formed by the small amount of dextrose present at the beginning of the experiment to start the accumulative reaction which by the end of the week had inverted nearly half of the sucrose, the invert content increasing from 1·14 to 21 % w/w on sample. At the same time the concentration had dropped from 49·5 % to 46 % w/w solids. As in raw sugar there is normally much less initial invert, the above reaction would take longer to reach completion. This also presumably happened with the second strain of yeast, which produced less acid and, therefore, less inversion. Quantitative or qualitative estimations of the acids formed have not yet been attempted.

CONCLUSION

Most of the organisms found growing in the film of molasses on sugar crystals belonged to the same sub-genus as osmophilic yeasts from similar sources. Those met with so far were not strict osmophiles but were tolerant of high concentrations of sugar. They also produced considerable quantities of acid, and were weakly fermentative.

It was not found possible to extract invertase from the yeasts chiefly examined, yet it was evident that sucrose was destroyed under natural conditions. The large amount of acid gradually produced explains this, as in weakly buffered solutions, the pH value steadily dropped, and during this process inversion by acid gradually took place. The second yeast, which appeared to produce less acid, showed less reaction. The thick surface ring produced in old cultures in concentrated syrups indicated that the process was mainly aerobic.

Comparing the growth reactions in 5 % and 54 % sucrose solutions, it was found that the optimum temperature was higher, the pH was lower, and the multiplication rate was much slower at the higher concentrations. Outside these ranges, a preliminary shrinkage of the whole cell, which always occurred after inoculation, was not overcome and the cells were apparently moribund.

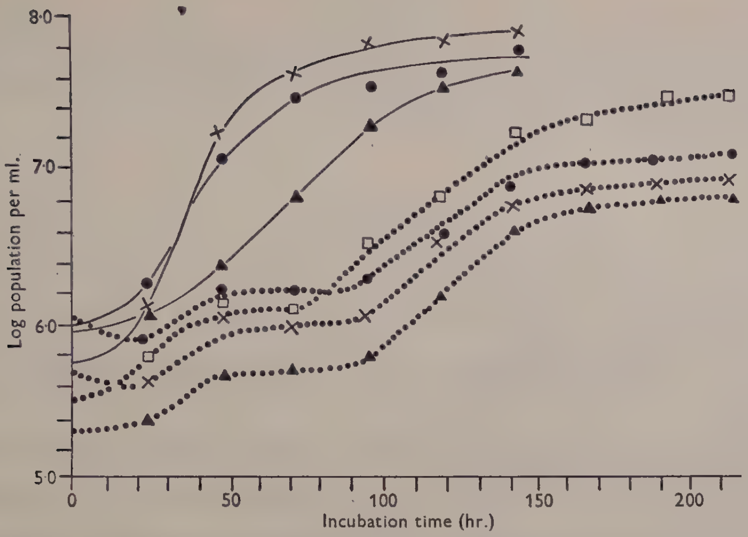

Fig. 4. Effect of amino-acids on growth rates of strain 21. *Zygosaccharomyces* sp. (40 μg. amino-acid per ml.) growing in 50 % w/w sucrose solutions. ——, with 1 % w/w added glucose; ••••••••••, without 1 % w/w added glucose; —×—×—, with added glutamine; —●—●—, with added isoleucine; —▲—▲—, with added aspartic acid; —□—□—, with added Yeastrel.

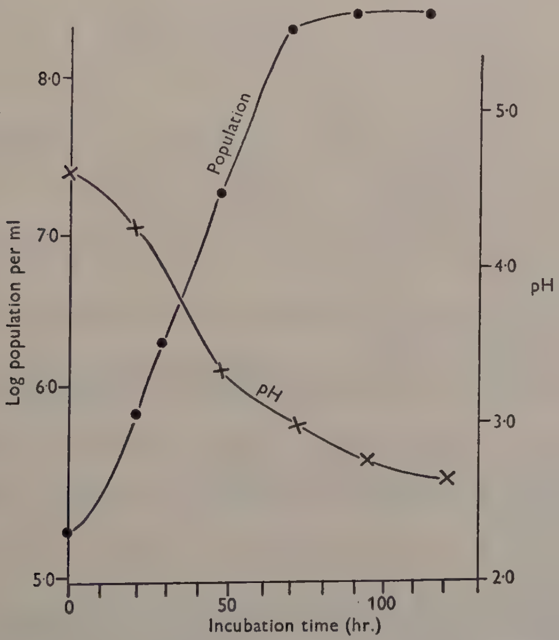

Fig. 5. Acid formation in complete medium containing dextrose (1 %) and sucrose (50 %) under gentle aeration. ——×——×——, pH change per unit time; ——●——●——, population change per unit time.

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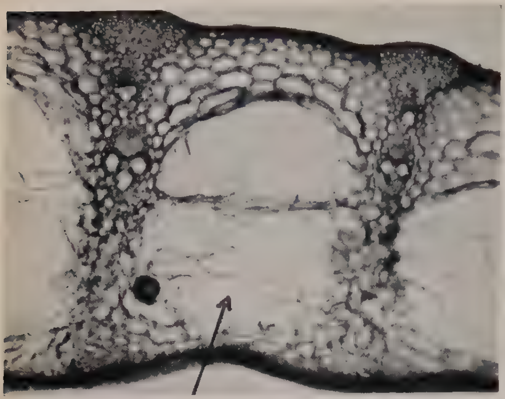

Fig. 1

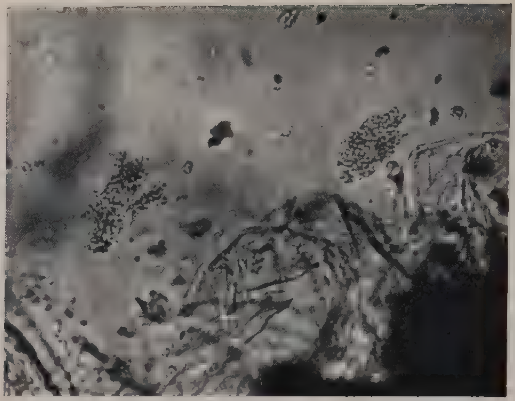

Fig. 2

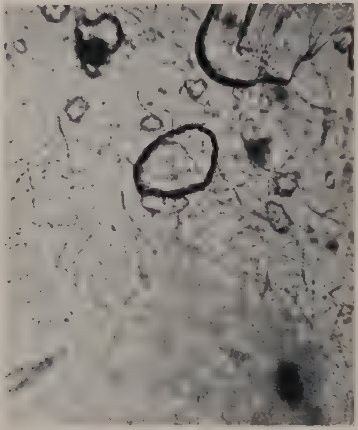

Fig. 3

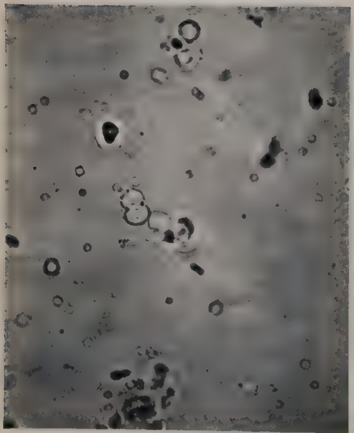

Fig. 4

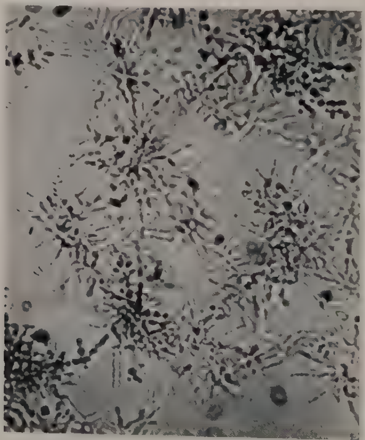

Fig. 5

M. Pamela Scarr—Osmophilic Yeasts in Raw Beet and Cane Sugars and Intermediate Sugar Refining Products. Plate 1

Whalley, H. C. S. de & Scarr, M. P. (1947). Micro-organisms in raw and refined sugars and intermediate products. *Chem. & Industr.* 351.

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EXPLANATION OF PLATE

Fig. 1. Transverse section, part of sheathing leaf base of sugar cane with arrow showing where most of the yeasts were found. ×37.

Fig. 2. Yeast growing in the region marked with arrow in Fig. 1; from dissection of fresh cane. ×120.

Fig. 3. Water mount of raw beet sugar showing contamination with hyphae of *Aspergillus glaucus*. ×90.

Fig. 4. Ascospore formation in 4-weeks' old wort agar streak of *Zygosaccharomyces* sp. isolated from Peruvian raw cane sugar. About 50% of the cells have formed asci; there was considerable drying out of the medium. ×900.

Fig. 5. Pseudomycelium formation from the sediment in a wort broth culture of the strain 21 *Zygosaccharomyces* sp. The formation develops in about 2 weeks. ×900.

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On the Nutrition of *Desulphovibrio desulphuricans*

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SUMMARY: A liquid medium based on peptone, yeast extract and glucose is described, which is capable of supporting massive growth of *Desulphovibrio desulphuricans* 'Hildenborough' and certain other strains of sulphate-reducing bacteria. When peptone is used in large amounts yeast extract may be omitted, though yeast extract is stimulatory when the peptone supply is suboptimal. Glucose is better than lactate in this medium. Bicarbonate increases the growth rate. More than one factor is responsible for growth stimulation by peptone and yeast extract; the factors responsible for the stimulation shown by yeast extract in the presence of inadequate peptone are water-soluble and not removed by various extraction procedures. Cysteine promotes growth, and serine, ornithine and isoleucine act synergistically with it. These amino-acids do not account for the whole activity of peptone and yeast extract.

The isolation and cultivation of sulphate-reducing bacteria were described by Baars (1930), Starkey (1938), Starkey & Wight (1945). The liquid media used by these workers were in general based on a carbon source such as lactic acid and inorganic salts, though Stephenson & Stickland (1931) used a meat broth nutrient agar and Starkey (1938) used a peptone glucose agar for the isolation of discrete colonies. Iya & Sreenivasaya (1945) showed that urea, peptone or casein hydrolysate stimulated the growth of a halophilic strain. Butlin, Adams & Thomas (1949) studied the growth of *D. desulphuricans* spp. in some detail and introduced the use of sodium sulphite for the purification of cultures; they also observed that the growth of pure cultures was stimulated by the addition of 0·1 % yeast extract. Butlin & Adams (1947) had previously shown that pure cultures were able to grow autotrophically.

Traditionally, these organisms have been cultivated in the presence of an excess of ferrous iron, their presence being inferred from the black precipitate of ferrous sulphide formed as a result of their metabolism. Butlin *et al.* (1949) showed that the greater part of this iron was unnecessary, but that, in media freed of iron by 8-hydroxyquinoline, traces were needed. Using reagents which had not been specially purified, together with yeast extract, however, 'rapid and abundant growth' was obtained without added iron and without blackening of the culture. A liquid medium containing peptone, yeast extract, glucose and salts which supported better growth with certain strains than any media previously used has been reported from this laboratory (Postgate, 1949; Report, 1949). Such growth occurred without precipitation of iron sulphide and the medium was used for the preparation of resting-cell suspensions. Miller (1949*a*, *b*) studied the total amounts of hydrogen sulphide produced by sulphate-reducing bacteria in closed vessels, using several different media. The amount of H_2S formed was greatly increased by addition of peptone, yeast extract, liver extract, potato juice, protein hydrolysates or milk.

Some studies on the factors responsible for the massive growth of *D. desulphuricans* in media based on peptone, yeast extract and glucose are reported here. The growth of the organism has been studied directly, since there is evidence (Postgate, unpublished) that the specific rate of sulphate reduction can vary according to the strain and the age of the culture. Consequently the rate of sulphide production or the precipitation of ferrous sulphide may not be a satisfactory measure of growth. Particular precautions were taken to ensure the purity of the strains used.

METHODS

Organisms. A strain of *Desulphovibrio desulphuricans* known as 'Hildenborough', isolated from Wealden clay taken from Hildenborough, Kent, England, in 1946, was the main organism used in this work. It was a typical mesophilic Gram-negative motile vibrio, and was normally grown at 30°. This and all but two of the other strains used were originally purified by cultivation and plating on media containing 3 % Na_2SO_3 (Butlin *et al.*, 1949). They were all checked at intervals during the work for aerobic and anaerobic contaminants; none was found.

Media. Stock cultures were maintained on the medium of Baars (1930) and subcultured monthly, but for routine work the organism was subcultured every three or four days in medium A, containing 'Bacto-Peptone' or 'Bacto-Tryptone' (Difco) 5 g.; yeast extract (Difco) 4 g.; Na_2SO_4, 1·5 g.; $MgSO_4 . 7H_2O$, 1·5 g.; glucose 10 g.; in 1 l. distilled water, pH 7·2. Media and solutions of substances to be added were sterilized by autoclaving for 10 min. at 10 lb./sq. in., except for media containing glucose, which were sterilized by steaming for 1 hr. on three successive days. Certain pure substances likely to be unstable to heat were sterilized by Seitz filtration; they are mentioned in the text.

Techniques. Cultures (5 ml.) of the organism in 6 in. × $\frac{3}{4}$ in. soft glass test-tubes were incubated in a McIntosh & Fildes anaerobic jar containing hydrogen and 5 % (v/v) CO_2, and the palladium catalyst of the jar was protected from H_2S-poisoning by a cotton-wool pad soaked in lead acetate and dried. For routine subculture 0·5 ml. of a grown culture was added to 5 ml. of the new culture, but for tests of growth-factor activity a washed inoculum was used. A grown culture (two or three days after inoculation) was centrifuged, resuspended in 0·8 % saline, and its turbidity adjusted to a standard value (100 μg. dried cells/ml.) using a calibrated direct-reading photoelectric colorimeter (The 'E.E.L.', Electroselenium Ltd., Harlow, Essex). This suspension was added to the culture medium to give a final dilution of 1/100. Such a large inoculum was desirable for quantitative work since, though growth occurred with a $1/10^9$ dilution of a grown culture, irregularities in growth rate sometimes occurred with dilutions greater than 1/100.

The extent of growth in a culture was usually judged by inspection, but, where necessary, quantitative values were obtained by estimating the turbidity in the 'E.E.L.' colorimeter. Using a blue filter ('E.E.L.' No. 303) which transmitted chiefly light of wavelength 480·0 mμ., the relationship between turbidity and dry wt. cells/ml. suspension was linear over a satisfactory range. Readings were taken in this range; where necessary the cultures were diluted.

RESULTS

Optimal pH and the effect of bicarbonate. The organism was not markedly sensitive to the initial pH, and grew well between values of about 6·3 and 8·6. More acid pH values depressed growth considerably. The *rate* of growth was more rapid at higher pH values such as 8·6, though the stationary population was reduced as compared with that obtained at pH values around 7.

The pH of a medium having an initial pH greater than about 7 tended, over 1–2 hr., to reach this value in an atmosphere of hydrogen + 5 % (v/v) CO_2, presumably owing to absorption of CO_2 as the HCO_3' and CO_3'' ions. Thus the enhanced growth rate shown by *D. desulphuricans* at high initial pH values could well have been due to a stimulation of growth by free HCO_3' ions. This view was tested by setting up a range of cultures of various pH values greater than 7·0 using known amounts of NaOH, and a comparable range of cultures containing the amount of $NaHCO_3$ which would be formed from the added NaOH were this hypothesis correct. The addition of $NaHCO_3$ had no significant effect on the initial pH of the medium, but, nevertheless, caused a stimulation of the growth rate equivalent to that obtained in the cultures containing NaOH. Thus free bicarbonate was preferable to gaseous CO_2 for optimal growth of the organism. For routine work, however, it was convenient to avoid the addition of bicarbonate to the cultures, so a pH value of 7·2 was finally adopted as giving the most satisfactory compromise between a good growth rate and a large stationary population.

The effect of the gas-phase. Medium A had growth-promoting activity in the presence or absence of hydrogen + CO_2 though growth was more rapid in its presence. The availability of gas determined the rate of growth of the cultures, since shallow cultures grew faster than deep ones (Fig. 1). It was thus necessary to use vessels of similar dimensions when making quantitative comparisons of bacterial growth.

The effect of peptone. When sufficient peptone was added, yeast extract could be omitted from the medium altogether. Either 'Bacto-Peptone' (Difco) or 'Bacto-Tryptone' (Difco) could be used but certain other proprietary peptones were less active. For optimal growth in the absence of yeast extract 20 mg./ml. of peptone were required (Fig. 2).

The effect of yeast extract. Yeast extract did not replace peptone satisfactorily since it depressed growth at high concentrations (Fig. 2). Growth with all concentrations of yeast extract appeared slower than with similar amounts of peptone but this effect was not studied in detail. With partially inhibitory concentrations of yeast extract the medium became darkened during growth, and allowance was made for this in obtaining the readings for Fig. 2.

The effect of peptone and yeast extract. A mixture of peptone and yeast extract exerted a greater growth-stimulating effect than either substance exerted separately, and was consequently preferable for routine subculture of the organism. Optimal growth was obtained using 5 mg./ml. peptone and 4 mg./ml. yeast extract (Fig. 3). The greater specific activity of a mixture

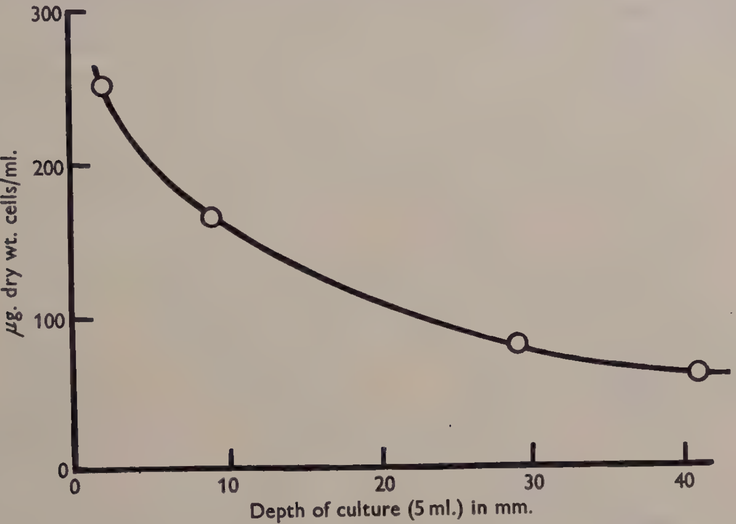

Fig. 1. Growth of *D. desulphuricans* 'Hildenborough' after 48 hr. in 5 ml. cultures of various depths.

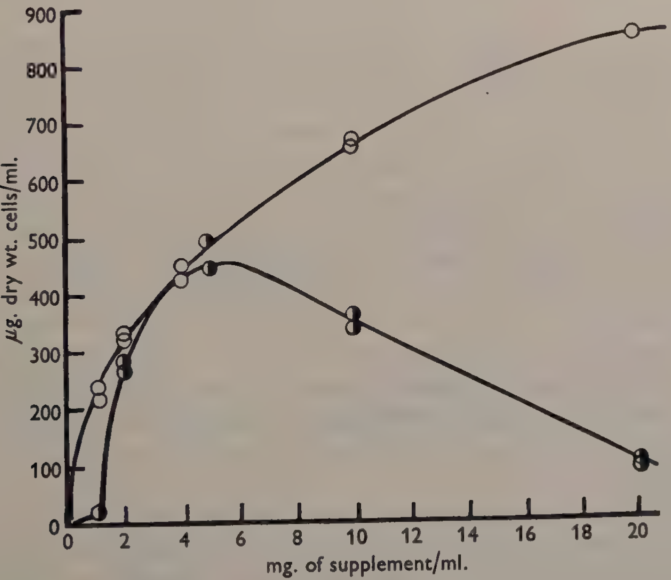

Fig. 2. Growth of *D. desulphuricans* 'Hildenborough' in a medium of glucose, salts and various supplements. ○ growth after 72 hr. with 'Bacto-Tryptone'; ◑ growth after 94 hr. with 'Difco' yeast extract.

of peptone and yeast extract compared with that of either used alone suggested that several factors were responsible for their stimulatory effect, some being more plentiful in yeast extract and others more plentiful in peptone.

The effect of other supplements. Casein hydrolysate (Ashe Laboratories, vitamin-free) was active in place of peptone and yeast extract, but growth with 20 mg./ml. of this material was markedly inferior to growth with 20 mg./ml. 'Bacto-Peptone' (Difco). Partial enzymatic digests of casein obtained by using trypsin were also active, and so were partial hydrolysates of casein prepared by using sulphuric acid.

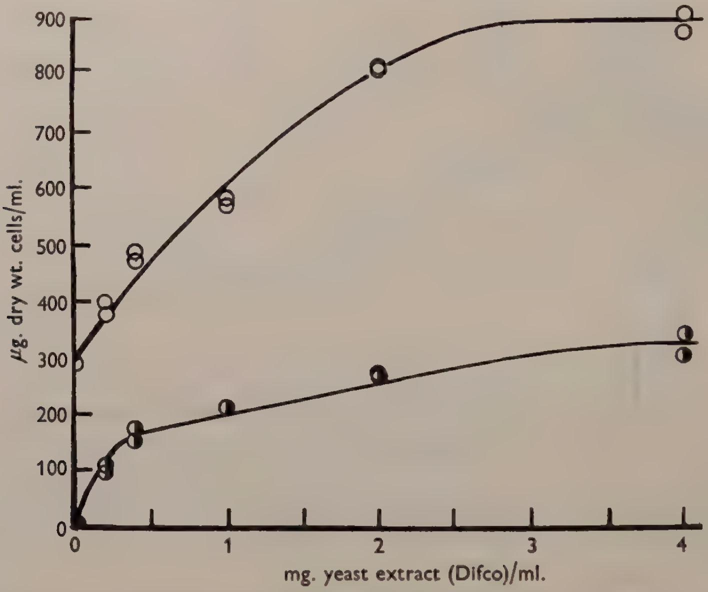

Fig. 3. Effect of yeast extract on the growth of *D. desulphuricans* 'Hildenborough' after 70 hr. ○ growth with 'Bacto-Peptone' (5 g./l.), glucose (10 g./l.) and salts; ◑ growth with lactic acid and salts (Starkey, 1938).

An extract prepared by boiling garden soil with an equal volume of water for 30 min. had no growth-promoting action, and an extract of fully-grown cells of *D. desulphuricans* prepared by boiling for 30 min. and centrifuging was also inactive.

Some studies with Starkey's medium. Growth in a medium consisting of lactic acid and salts (Starkey, 1938) was shown to be stimulated by 1 mg./ml. yeast extract (Butlin *et al.*, 1949). Fig. 3 shows that greater quantities of yeast extract stimulated growth further, but not to the level obtained with glucose and peptone.

Glucose was an important constituent of the new medium since cultures in Starkey's medium enriched with 0·1 % (w/v) 'Bacto-Tryptone'+0·2 % (w/v) yeast extract gave only 420 μg. dry wt. of cells/ml. after 72 hr. incubation, as compared with 710 μg. dry wt. of cells/ml. obtained in parallel cultures with the same supplements+glucose (1 % w/v) and salts. In both media the carbon sources were present in excess of the normal requirement of the organism.

Some tests with other strains of D. desulphuricans. Massive growth in medium A was not shown by all strains of sulphate-reducing bacteria, though growth was usually superior to that obtained in lactic acid + yeast extract media. A test using strains usually subcultured at 30° is recorded in Table 1, where the growth in medium A was compared with the growth in Starkey's lactic acid medium with 0·1 % (w/v) yeast extract added as used by Butlin *et al.* (1949). The inoculum for this test was prepared from a culture grown in medium A, so if an adaptive process were involved in the response to these media the differences shown in Table 1 are exaggerated in favour of medium A. The results, nevertheless, distinguish between those strains which give a considerable response to the new medium and those which do not.

Table 1. *Growth of* D. desulphuricans *in various media*

Strain	μg. of dried cells/ml. in peptone-yeast-glucose medium	μg. of dried cells/ml. in lactate-yeast-salts medium	Hours of Incubation
Wandle	500	70	120
Teddington M	190	60	120
Teddington R	360	135	120
**Texas 29.12.B	400	190	96
Essex 6	425	115	120
Hildenborough	500	120	120
*New Jersey, Sol C-1	240	180	96
*Holland, D-6	210	150	96
*Holland, SH-1	230	170	96

* Kindly supplied to this laboratory by Dr Starkey.

** A halophil grown in 3 % NaCl; kindly supplied to this laboratory by Dr ZoBell.

An inconsistency has been noted in the behaviour of some strains. On the first occasion of testing 'Essex 6' showed little stimulation of growth in the new medium, though some months later (after a few subcultures in Baars's medium) the effect recorded in Table 1 was observed. Similarly 'Teddington R' (a strain distinguished from all others yet isolated by being non-motile), when first tested showed as remarkable an effect as 'Hildenborough', though after a few passages on Baars's medium the effect had diminished. The strain was morphologically unchanged. On yet another occasion 'Hildenborough' lost its ability to grow profusely in medium A, though it had been subcultured for 90 passages in this medium, regularly giving massive growth. A new series from the stock culture in Baars's medium behaved satisfactorily. The reason for these irregularities is unknown; in the last-mentioned case the alteration was not due to any change in the batch of medium, the brand or batch of peptone, yeast extract or glucose, or to the atmosphere, the pH of the medium or the inoculum size. The culture was uncontaminated and morphologically unaltered.

Some chemical tests on yeast extract. Though there were probably several factors responsible for the stimulation of growth obtained with peptone and yeast extract, it was nevertheless likely that all of these were present in peptone, as large amounts of peptone would support optimal growth in the absence of yeast extract. With insufficient peptone it was likely that yeast extract in stimulating growth was acting by means of a factor or factors not plentiful in peptone. Since in these conditions the number of factors experimentally sought was decreased, tests on the chemical behaviour of yeast extract (Difco) were undertaken using medium A without yeast extract as a test medium. The following points were established:

(1) The factors in yeast extract were stable to heat. The material could be sterilized in steam for 10 min. at 10 lb./sq. in. and when this was increased to 20 min. at 20 lb./sq. in. there was no loss of activity.

(2) A distillate from the pyrolysis of dry yeast extract was inactive.

(3) Activity remained exclusively in the residue after extraction of yeast extract with chloroform, benzene, 60–80° ligroin, acetone and ethanol.

(4) Activity remained exclusively in the aqueous phase after continuous extraction of aqueous solutions at pH 3, 6, 9 and 12 with diethyl ether.

(5) Aqueous solutions treated with excess of acetone or ethanol gave a precipitate which contained the active factors.

(6) Neither animal charcoal nor 'Brockman' alumina removed the active factors from a neutral aqueous solution.

Similar tests were not in general carried out with peptone owing to the complex nature of its activity. Peptone, however, remained active after autoclaving for 10 min. at 10 lb./sq. in., and its activity was lost on ashing. This last test indicated that the activity of peptone was not due to the presence of non-volatile inorganic constituents.

Some tests of substances having biological activity. A number of pure biologically-active substances was tested for ability to replace peptone and yeast extract in a medium containing glucose (10 g.), Na_2SO_4 (1·5 g.), and $MgSO_4.7H_2O$ (1·5 g.), in 1 l. Since there was a deficiency of phosphate and inorganic nitrogen, which might have led to a failure to detect activity with a pure substance, 1 g. $NH_4.H_2PO_4$/l. was added. It was desirable to sterilize the phosphate separately as in its presence some caramelization of the medium occurred during steam sterilization. A washed inoculum of *D. desulphuricans* 'Hildenborough' was used for all tests. The test substances, except for those mentioned, were sterilized by autoclaving for 10 min. at 10 lb./sq. in.

The compounds were tested singly, in groups, and finally as a mixture. After four or five days limited growth usually occurred in the basal medium, and where this growth was inhibited the compound was retested. This phenomenon occurred once only, when 2-methyl-1 : 4-naphthaquinone depressed growth slightly at 10 μg./ml. The compounds and the highest concentrations tested are listed in Table 2.

Most of these compounds are of well-known biological importance, but some may require explanation. Carotene and β-indoleacetic acid are not known to be important in the metabolism of micro-organisms: a report that the latter

Table 2. *Substances tested as growth factors for* D. desulphuricans *'Hildenborough', and the highest test concentrations used*

Vitamins		*Amino-acids*	
p-Aminobenzoic acid	100 mμg./ml.	DL-Alanine	1 mg./ml.
Aneurin	100 μg./ml.	L-Arginine	1 mg./ml.
Ascorbic acid	100 μg./ml.	DL-Aspartic acid	1 mg./ml.
B_{12}**	4 mμg./ml.	L-Cysteine*	2 mg./ml.
Biotin**	100 mμg./ml.	L-Cystine	saturated
Carotene	saturated	L-Glutamic acid	1 mg./ml.
Choline*	100 μg./ml.	DL-Glutamic acid	1 mg./ml.
Haemin	saturated	L-Histidine	1 mg./ml.
m-Inositol	100 μg./ml.	L-Hydroxyproline	1 mg./ml.
Nicotinic acid	100 μg./ml.	L-Leucine	1 mg./ml.
Nicotinamide	100 μg./ml.	DL-Isoleucine	1 mg./ml.
Oleic acid	saturated	DL-Norleucine	1 mg./ml.
Pantothenic acid	100 μg./ml.	DL-Lysine	1 mg./ml.
Pteroylglutamic acid	100 mμg./ml.	DL-Methionine*	1 mg./ml.
Pyridoxine*	100 μg./ml.	DL-Ornithine	1 mg./ml.
Pyridoxal*	100 μg./ml.	DL-Phenylalanine	1 mg./ml.
Pyridoxamine*	100 μg./ml.	L-Proline*	1 mg./ml.
Riboflavine*	100 μg./ml.	DL-Serine	1 mg./ml.
		DL-Threonine	1 mg./ml.
Miscellaneous		DL-Tryptophan*	1 mg./ml.
Ammonium acetate	1 mg./ml.	DL-Tyrosine	saturated
Asparagine	1 mg./ml.	DL-Valine	1 mg./ml.
Glutamine*	1 mg./ml.	DL-Isovaline	1 mg./ml.
Glutathione*	1 mg./ml.		
β-Indoleacetic acid	10 μg./ml.		
2-methyl-1:4-naphthaquinone	1 μg./ml.	*Purines, etc.*	
		Adenine	1 mg./ml.
Putrescine	5 μg./ml.	Allantoin	1 mg./ml.
'Reductone'*	1 mg./ml.	Guanine	saturated
Sodium bicarbonate	5 mg./ml.	Thymine	1 mg./ml.
'Tween 80'	100 μg./ml.	Uracil	1 mg./ml.
Urea*	10 mg./ml.	Xanthine	saturated

* Sterilized by Seitz-filtration.
** Obtained sterile.

promoted the growth of *Escherichia coli* bacteriophage (Cohen & Fowler, 1947*a*) has been withdrawn (Cohen & Fowler, 1947*b*). It was, nevertheless, thought desirable to test them since they were available. 2-Methyl-1:4-naphthaquinone has vitamin K activity for certain organisms (Knight, 1945) and was used in place of phthiocol which was not available. 'Reductone' (CHO . C(OH) : CHOH) is a compound which may be important in the biosynthesis of the pterin group of factors (Forrest & Walker, 1948) and, since it is also formed by the partial oxidation of glucose (O'Meara, McNally & Nelson, 1944, 1947), any activity found could account for the importance of glucose in the medium. Ammonium acetate was tested since acetate has been shown to be important for the growth of certain organisms (e.g. Collins, Nelson & Parmelee, 1950) and also to ensure that the basal medium was not merely deficient in nitrogen as NH_4^+. Sodium bicarbonate was tested since it has already been shown to increase the growth rate in rich media, and ascorbic acid, though not a growth factor for bacteria, was tested because its powerful reducing properties might assist the growth of an anaerobe. Urea was tested for two reasons: firstly, Iya & Sreenivasaya (1945) showed that it accelerated sulphide formation by a halophilic strain of *D. desulphuricans*, and secondly, it is biologically related to ornithine through the cycle proposed by Krebs for the formation of urea in tissues. Ornithine was one of the materials found to be active.

In view of the inactivity of peptone ash it was unlikely that the activity of peptone was due to its supplying traces of inorganic materials to the organism unless these were volatile. To confirm this view tests were carried out on a solution containing the following elements such that the concentration of each constituent was between 1 and 10 μg./ml.: Fe^{++}, Fe^{+++}, Al^{++}, Cu^{++}, Mg^{++}, UO_2^{++}, Zn^{++}, Mn^{++}, As, Si, B, Mo and V (the last five as oxy-acid anions). This mixture was inactive both alone and with a suboptimal amount of peptone (5 %, w/v).

Of the materials listed in Table 2 only cysteine was active in the first series of tests, and an optimal concentration of 2 mg./ml. was established. The tests were repeated in the presence of this material, and serine, ornithine and isoleucine were found to have synergistic activity with cysteine. A mixture of these four amino-acids (cysteine 2 mg./ml., serine 1 mg./ml., ornithine 1 mg./ml. and isoleucine 1 mg./ml.) was able to support growth up to 1/5 of that obtained with optimal amounts of peptone. This is indicated in Table 3.

Table 3. *Growth of* D. desulphuricans *after* 96 *hr. with various amino-acids*

Added to glucose (1 % w/v)+salts medium (cysteine at 2 mg./ml., others at 1 mg./ml.)	Growth in μg. of dried cells/ml. 'Hildenborough'	 'Essex 6'
No addition	2	2
Cysteine	45	62
Cysteine+serine	87	76
Cysteine+serine+ornithine	117	100
Cysteine+serine+ornithine+isoleucine	126	138
Peptone (5 g./l.)+yeast extract (4 g./l.)	750	224

These amino-acids were also active towards *D. desulphuricans* strain 'Essex 6', though at the time of testing this strain did not show the remarkable stimulation of growth in peptone yeast extract glucose medium found with 'Hildenborough' (or with 'Essex 6' at a later date—see above) (Table 3).

With 'Hildenborough' strain, growth with the four amino-acids at the concentrations mentioned was not improved by the following expedients:

(1) Doubling the amino-acid concentration.

(2) Doubling the concentration of each active amino-acid in turn.

(3) Decreasing the concentration of each amino-acid in turn to 40 % of the values mentioned.

(4) Adding all the compounds listed in Table 2 (excluding ammonium acetate, urea, and sodium bicarbonate) either separately or as a mixture.

(5) Adding yeast extract at a concentration (1 g./l.) active when the amino-acids are replaced by a suboptimal amount (5 g./l.) of peptone.

It was, therefore, likely that substances other than those tested were present in peptone and yeast extract and were mainly responsible for their activity; the four active amino-acids might be present and might contribute to the activity.

DISCUSSION

Of the amino-acids cysteine, ornithine, serine and isoleucine, which stimulated growth, cysteine was the most important, since the other amino-acids were not active in its absence. It is probable that ornithine, serine and isoleucine were acting in their own right rather than through an impurity of biological activity since they were of synthetic origin. No specimen of synthetic cysteine was available for testing, so the possibility remains that its activity was due to impurity.

These four amino-acids may be present in peptone and may contribute to its activity, but the evidence was against their being responsible for the whole of its activity. They supported considerably less growth than peptone, and this limited growth was not due to antagonisms among the active amino-acids, since alteration of their ratios effected no improvement. On the other hand, they were the racemic mixtures of the amino-acids: the laevo-isomers, had they been available, might have shown greater activity. However, the fact that they were active for a strain of *D. desulphuricans* which showed little stimulation by peptone suggests that they were not responsible for the greater stimulation which occurred with the test strain. It is, therefore, likely that at least one other factor was responsible for the activity of peptone.

It is possible that the missing factor or factors were of the polypeptide group, since peptone is likely to be rich in such compounds, as were the partial tryptic digests of casein which were also active. Sprince & Woolley (1945) have shown that a substance of this nature, strepogenin, is required for the growth of certain lactic bacteria. No preparation of strepogenin was available for testing, but the partial tryptic casein digests were prepared so as to conserve this material as much as possible (Dr Woolley, personal communication). Smith & Douglas (1950) have also reported an unidentified factor necessary for the growth of *Clostridium bifermentans*, which they believe to be a peptide other than strepogenin. This is present in peptone preparations and partial digests and hydrolysates of proteins, and its solubility properties resemble those of the yeast-extract component studied here.

Rittenberg (1941) found that peptone lactate agar was as effective as peptone glucose agar for the growth of marine sulphate-reducing bacteria, but in the present studies glucose was necessary for the full growth-promoting effect of medium A. The reason for this is not known, though formation of reductone was shown to play no part in the effect.

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The Reduction of Sulphur Compounds by *Desulphovibrio desulphuricans*

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SUMMARY: *Desulphovibrio desulphuricans*, strain 'Hildenborough', was able to use sulphite, thiosulphate, tetrathionate, metabisulphite or dithionite in place of sulphate for growth. Resting cell suspensions reduced these ions using the theoretical amounts of hydrogen and forming the theoretical amount of sulphide, except in the case of dithionite, which probably decomposed spontaneously to sulphate and sulphur before being reduced.

The organism was unable to grow with or to reduce dithionate, perdisulphate, 'formaldehydesulphoxylate', sulphamate, benzenesulphonate, methanesulphonate, β-hydroxyethane-sulphonate, sodium ethylsulphate, dimethylsulphone or cystine. Elementary sulphur, if purified by re-distillation, was also not attacked. Five other strains of *D. desulphuricans*, four of them cultivated autotrophically, were also unable to grow with pure elementary sulphur.

Colloidal sulphur permitted slow growth, or slow hydrogen absorption when a resting cell suspension was used. This effect was not due to oxide impurities in the sulphur permitting growth, as an ultra-filtrate of colloidal sulphur had considerably less activity.

A study of the rates of H_2 uptake suggested that sulphur, thiosulphate and tetrathionate were not intermediates in normal sulphate reduction, but that sulphite was.

The sulphate-reducing bacteria were first described by Beijerinck (1895), and were subsequently investigated by many workers including van Delden (1903), Baars (1930), Starkey (1938), Butlin & Adams (1947), ZoBell & Rittenberg (1948) and Butlin, Adams & Thomas (1949 *b*). 'Growth' was usually estimated by the amount of black ferrous sulphide precipitate formed by reaction of the sulphide produced from the reduction of sulphate with the excess of ferrous salt added to the medium. Butlin, Adams & Thomas (1949 *b*) showed that this large amount of iron is unnecessary, though traces insufficient to form a visible precipitate of iron sulphide are needed for growth.

The media used for the cultivation of these organisms were based on the use of a carbon source such as lactic acid, glucose or, for growth in autotrophic conditions, bicarbonate. Except in the latter case the carbon source acted also as a hydrogen donor for sulphate reduction, and the ability of various organic compounds to act as hydrogen donors was studied by Baars (1930). Butlin & Adams (1947) showed that, in autotrophic media, mild steel could act as a hydrogen donor, due to the formation of an electrolytic film of hydrogen at its surface in contact with the medium. Stephenson & Stickland (1931) showed that a strain of this organism was able to utilize gaseous hydrogen, and that sulphate reduction proceeded quantitatively according to the equation:

$$SO_4'' + 4H_2 = S'' + 4H_2O.$$

Postgate (1949) reported similar results with washed cell suspensions which reduced sulphate, sulphite and thiosulphate. Baars (1930) found that sulphate-reducing bacteria would grow with sulphite, thiosulphate, 'hydrosulphite' (dithionite) or colloidal sulphur in place of sulphate, but not with 're-precipitated' sulphur. When studying marine halophilic strains ZoBell & Rittenberg (1948) added tetrathionate to this list.

Butlin, Adams & Thomas (1949*b*) showed that growth and sulphide formation occurred with sulphate, sulphite, thiosulphate, and, in contrast to Baars (1930), also with 're-precipitated' sulphur, in autotrophic as well as heterotrophic conditions.

Evaluation of growth by sulphide formation may be unsatisfactory, since sulphide formation can take place without cell division, and this technique has probably been used only because of its ease and convenience. In media based on lactic acid and salts, growth without added iron is not abundant, but it is improved by the addition of yeast extract (Butlin, Adams & Thomas, 1949*b*). Recently, a medium giving vigorous growth without precipitation of iron sulphide has been described (Postgate, 1951) and used to grow large amounts of cells for manometric studies (Postgate, 1949). Using a sulphate-free modification of this medium, one can observe the growth of the organisms directly and investigate the ability of various compounds to replace sulphate for growth. Some studies of this kind are reported below, together with a quantitative investigation of the reduction of certain sulphur compounds by resting cell suspensions.

METHODS

Organism. A strain of *D. desulphuricans* called 'Hildenborough' was used in this work except where otherwise stated. Its origin and the routine methods of maintenance were described by Postgate (1951). Washed inocula were used for all growth tests recorded here.

Media and Materials. To study the ability of substances to replace sulphate it was desirable to have a medium which permitted optimal growth in the presence of sulphate and none in its absence. Such a medium was not easily prepared because most peptone preparations contain sulphate. A medium was found, however, based on Difco 'Bacto-Tryptone' (Baird & Tatlock Ltd., London), which permitted only very limited growth in the absence of added sulphate, owing probably to its low content of inorganic sulphur ('Difco Manual', 1948). This medium contained 'Bacto-Tryptone', 5 g.; glucose, 10 g.; yeast extract (Difco), 4 g.; $MgCl_2 . 6H_2O$, 0·8 g., in 1 litre of distilled water; pH 7·2. This will be referred to as the *complex medium.*

For certain tests it was necessary that no growth at all should occur in the absence of added substrate, and for this purpose a modification of Starkey's medium (Starkey, 1938) was used, having the composition: KH_2PO_4, 0·5 g.; NH_4Cl, 1 g.; $CaCl_2 . 6H_2O$, 0·1 g.; $MgCl_2 . 6H_2O$, 1·1 g.; sodium lactate (British Drug Houses Ltd., 70 % w/w) 5·0 g., in 1 litre of distilled water; pH 7·5. This medium gave only limited growth in the presence of sulphate owing to the absence of complex organic supplements. It will be referred to as the *simple medium.*

Where possible, materials of 'Analar' grade were used, but in certain cases (mentioned in the text) such specimens were not available. Sodium tetrathionate was at first prepared from an ethanolic solution of iodine and sodium thiosulphate, using the method described by Partington (1946), and was reprecipitated twice with absolute ethanol before use. This procedure did not give a pure preparation, as is mentioned later. 'Analar' grade $Na_2SO_3 . 7H_2O$ was recrystallized.

Substances to be tested were usually sterilized by autoclaving for 10 min. at 10 lb./sq. in., but when the substances might be unstable to heat they were added without sterilization. Such cases are mentioned; at the end of the test the cultures were always examined for contaminants, but none were found.

Resting Cell Suspensions. Cell suspensions for use in the Warburg manometer were prepared by centrifuging a culture and resuspending the cells in aqueous NaCl (0·8 % w/v) so as to give a suspension containing between 2 and 3 mg. dry wt. of cells/ml. Traces of substrate carried through the washing procedure were eliminated by pre-incubating the suspension for about 20 min. in a stream of hydrogen before use. One ml. of this suspension was added to the manometer vessel with 1 ml. of KH_2PO_4 buffer (0·5 % w/v, pH 6·3) and the substrate (usually 2·5 μmol.) was added in 0·5 ml. of water to the side arm; 0·5 ml. of aqueous NaOH (15 % w/v) or cadmium chloride (10 % w/v) were contained in the centre cup together with filter paper or glass wool. The manometer vessels were incubated at 37°. Six determinations of the $-Q_{H_2}$ for sulphate with a certain suspension gave a mean value of 323 mm^3 H_2/mg. dry cells/hr. with a standard deviation of 29·25 mm^3/mg./hr.; five determinations of the hydrogen absorbed with *c.* 2·5 μmol. of Na_2SO_4 gave a mean absorption of 276·6 mm^3 H_2 with a standard deviation of 2·72 mm^3 H_2.

Sulphide Estimations. Attempts to estimate the sulphide formed in the manometer vessel by analysis of the NaOH-soaked paper in the centre cup were unsuccessful, but a method using cadmium chloride in place of NaOH, and glass wool in place of filter paper proved satisfactory and was used throughout this work.

The manometer vessels were set up with a pre-incubated cell suspension and phosphate buffer in the main compartment and substrate in the side-arm. A small plug of acid-cleaned glass wool was placed in the centre cup and 0·5 ml. of aqueous $CdCl_2$ (10 % w/v) was added. At the end of the experiment the plug was transferred with clean tweezers to a stoppered titration vessel containing 1 ml. of 0·1 N iodine and 4 ml. of 5 N-HCl, which was shaken and left for 5 min. The centre cup was then rinsed with the titration liquid, followed by distilled water, to remove the last traces of sulphide, and the washings were added to the titration vessel. The whole volume was back-titrated against 0·01 N sodium thiosulphate, using a drop of carbon tetrachloride as an internal indicator. This method gave satisfactory results down to about 0·3 μmol. of sulphide, provided the titration fluid was left until all

CdS had decomposed (5 min.), and provided the titration vessel was kept stoppered as much as possible to prevent back-oxidation of HI. The standard deviation calculated from 12 readings taken over some months was 0·1273 μmol. of sulphide. The use of $CdCl_2$ in place of NaOH in the manometer made no difference to the amount of gas absorbed with a given substrate, indicating that no CO_2 is formed during sulphate reduction and that no correction need be applied for this.

RESULTS

Growth Experiments

Without substrate. No growth occurred in sulphate-deficient simple medium, but some growth occurred in the sulphate-deficient complex medium. This 'blank' growth was probably due to sulphate and other reducible compounds present in the 'Bacto-Tryptone' and yeast extract; indeed, the former is known to contain 0·04 % of inorganic sulphur ('Difco Manual', 1948).

Sulphate. Sulphate is the natural hydrogen-acceptor for the growth of *D. desulphuricans*. In the sulphate-deficient complex medium the growth of the organism depended linearly on the sulphate concentration between about 0·5 and 10 μmol. sulphate/ml. Higher concentrations of sulphate depressed the growth rate to some extent, though the maximum stationary population was ultimately reached (Fig. 1). Optimal growth was given by 10 μmols sulphate/ml., and this concentration was therefore used for testing other compounds, except in the case of elementary sulphur (see below).

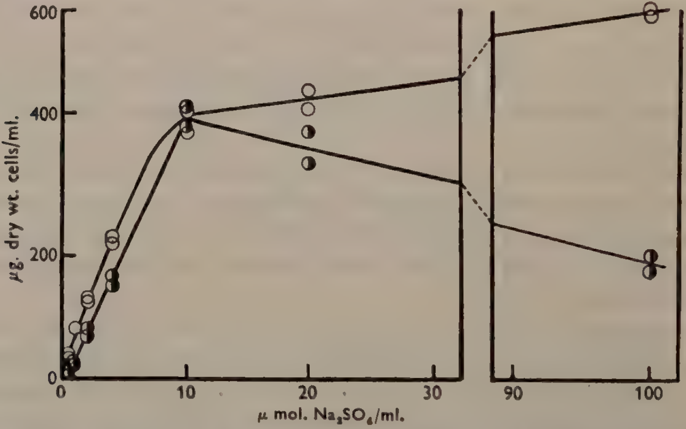

Fig. 1. Effect of sodium sulphate concentration on the growth of *D. desulphuricans* (Hildenborough) in a medium of glucose tryptone and yeast extract. ○ growth after 142 hr. incubation; ◑ growth after 92 hr. incubation.

Sulphite. Sodium sulphite could replace sulphate for the growth of *D. desulphuricans* in the sulphate-deficient complex medium (Table 1). It has been used in other media, at a concentration of 3 %, to purify crude cultures (Butlin, Adams & Thomas, 1949*b*) and has been observed to cause considerable elongation of the cells (Butlin, Adams & Thomas, 1949*a*).

Thiosulphate. Sodium thiosulphate could replace sodium sulphate for growth of the organism in the sulphate-deficient complex medium (Table 1).

Tetrathionate. Sodium tetrathionate could replace sodium sulphate for growth of the organism in sulphate-deficient complex medium, but growth took place more slowly (Table 1).

Table 1. *Growth of* D. desulphuricans (*Hildenborough*) *on a medium of glucose, peptone and yeast extract containing various substances in place of sulphate*

	Concentration	Growth after (hr.)			
Test substance	M	24	48	72	96
Sulphate Na_2SO_4	10^{-2}	tr.	+	+++++	+++++
Sulphite Na_2SO_3	10^{-2}	tr.	+	+++++	+++++
Thiosulphate $Na_2S_2O_3$	10^{-2}	tr.	+	+++	+++++
Dithionate $Na_2S_2O_6$	10^{-2}	—	tr.	tr.	tr.
*Tetrathionate $Na_2S_4O_6$	10^{-2}	tr.	tr.	++	++++
*Dithionite $Na_2S_2O_4$	10^{-2}	—	tr.	+++	++++
Perdisulphate $(NH_4)_2S_2O_8$	10^{-2}	—	tr.	tr.	tr.
*Formaldehyde sulphoxylate $NaHSO_2 . HCHO$	10^{-2}	—	tr.	tr.	tr.
*Metabisulphite $K_2S_2O_5$	10^{-2}	tr.	tr.	++	+++
Benzenesulphonate $NaSO_3 . C_6H_5$	10^{-2}	—	tr.	tr.	tr.
Sulphur (B.D.H., re-ppt.) S	‡4×10^{-2}	—	tr.	+	+
*Sulphamate $NH_4 . SO_3NH_2$	10^{-2}	—	tr.	tr.	tr.
Methanesulphonate $NaO . SO_3 . CH_3$	10^{-2}	—	tr.	tr.	tr.
β-Hydroxyethanesulphonate $NaO . SO_2 . CH_2CH_2OH$	10^{-2}	—	tr.	tr.	tr.
†Sulphide Na_2S	10^{-2}	tr.	tr.	tr.	tr.
No addition	...	—	tr.	tr.	tr.
Sulphate Na_2SO_4	10^{-2}	—	tr.	+++++	+++++
†Ethylsulphate $Na(C_2H_5)SO_4$	10^{-2}	—	—	—	tr.
Dimethylsulphone $(CH_3)_2SO_2$	10^{-2}	—	—	—	tr.
No addition	...	—	—	—	tr.

* Added without sterilization. † Seitz filtered. ‡ Added as suspension.

Dithionite. Sodium dithionite ('hydrosulphite' British Drug Houses Ltd.) replaced sulphate for the growth of the organism (Table 1), but it is likely that at the test pH value of 7·2, most of the S_2O_4'' ion had decomposed spontaneously. Solutions of the specimen used decomposed slowly in water with deposition of sulphur, and were therefore made up immediately before use and added without sterilization. Nevertheless, within 1 hr. a precipitate of sulphur was visible in the test medium, and it is likely that sodium dithionite supported growth because it decomposed spontaneously to sulphate.

Metabisulphite. It was thought desirable to test potassium metabisulphite since the S_2O_5 is known to exist as a separate ionic species in solution, though whether it was stable in the test conditions was not known. It replaced sulphate for growth of the organism (Table 1), but growth took place slowly and was less extensive.

Sulphur. 'Re-precipitated' sulphur (British Drug Houses Ltd.) supported only limited growth of the organism, though a suspension of 40 μg. atoms sulphur/ml. was used for testing in contrast to the 10 μmol./ml. solutions used of other substances (Table 1). This low activity might have two causes: (*a*) the low solubility of the substrate or (*b*) inactivity of the sulphur *per se* masked by the presence of active impurities. To test the second possibility a specimen of sulphur was distilled in an all-glass apparatus with a stream of oxygen-free nitrogen, ground in a flamed mortar and tested at once, without further sterilization. It did not support the growth of *D. desulphuricans*, and, since the preparation was perfectly satisfactory for the aerobic growth of *Thiobacillus thio-oxidans*, this failure was not due to a change in the allotropic state of the sulphur such as to render it biologically inactive.

Since the inactivity of purified sulphur contrasted with the reports of previous workers, it was studied in more detail. Cultures were set up with excess iron ($FeCl_2$, 0·5 mg./ml.), and also without, in sulphate-free simple medium and in sulphate-deficient complex medium. In the simple medium neither growth nor blackening occurred with the purified sulphur, and in the complex medium growth and blackening were equivalent to that obtained in the cultures without added substrate (Table 2). A test of the same kind using *D. desulphuricans* strain 'Teddington R' gave a similar result.

Table 2. *Growth and sulphide formation of* D. desulphuricans (*Hildenborough*) *with sulphate and re-distilled sulphur* (1 *mg./ml.*)

With sulphate-deficient complex medium	Effect after (hr.)				
Addition	24	48	72	96	114
Sodium sulphate	—	++	++++	+++++	+++++
Re-distilled sulphur	—	—	tr.	tr.	tr.
—	—	—	tr.	tr.	tr.
Sodium sulphate + $FeCl_2$	*	*	*	*	*
Re-distilled sulphur + $FeCl_2$	—	—	*	*	*
$FeCl_2$	—	—	*	*	*
With sulphate-deficient lactate medium					
Sodium sulphate	—	+	++	+++	+++
Re-distilled sulphur	—	—	—	—	—
—	—	—	—	—	—
Sodium sulphate + $FeCl_2$	*	*	*	*	*
Re-distilled sulphur + $FeCl_2$	—	—	—	—	—
$FeCl_2$	—	—	—	—	—

* Sulphide formation detected by precipitation of FeS from $FeCl_2$ (0·5 mg./ml.) added to the culture medium.

Four strains cultured in autotrophic medium (Butlin, Adams & Thomas, 1949*b*) were also tested for their ability to utilize freshly-distilled sulphur, using the sulphate-deficient simple medium in which lactic acid was replaced by sodium bicarbonate. They were all tested in the presence of $FeCl_2$ (0·5 mg./ml.) so that growth was indicated by blackening of the culture. Since it was not convenient to wash the inocula, the inoculum cells for the test were grown with 're-precipitated' sulphur in place of sulphate. In no case was growth or blackening detected in the presence of freshly-distilled sulphur. A specimen of re-distilled sulphur which had been left in air for about twenty days gave detectable blackening in autotrophic cultures.

It was possible that the sulphate-reducing bacteria were able to utilize sulphur only in the colloidal form—which was the form used by Baars (1930)—so a specimen of colloidal sulphur was obtained for testing in both simple and complex media. It supported the growth of both 'Hildenborough' and 'Teddington R' strains in these media. The specimen was known, however, to contain polythionic acid impurities equivalent to 0·0154 % $H_2S_5O_6$, and the activity of the preparation may have been due to these. An ultra-filtrate of the sulphur sol was obtained by passing it through a 30–55 mμ. A.P.D. collodion membrame under 2 atm. N_2 pressure, and this also was able to support growth of the bacteria, though it was optically free from sulphur. These findings are illustrated in Table 3. In some experiments the ultra-filtrate supported distinctly less growth than the sol, but quantitative comparisons were most unreliable since the growth was judged by blackening due to FeS precipitation. Direct comparisons of the growth obtained with the sulphur sol and its ultra-filtrate could not be made owing to the opacity of the former.

Table 3. *Sulphide formation by* D. desulphuricans (*Teddington R*) *with colloidal sulphur and its ultra-filtrate*

With sulphate-deficient complex medium	Effect after (hr).			
Addition	24	48	72	96
Sodium sulphate + $FeCl_3$	*	*	*	*
Colloidal sulphur + $FeCl_3$	—	*	*	*
Ultra-filtrate + $FeCl_3$	—	*	*	*
With sulphate-deficient lactate medium				
Sodium sulphate + $FeCl_3$	—	*	*	*
Colloidal sulphur + $FeCl_3$	tr.	*	*	*
Ultra-filtrate + $FeCl_3$	tr.	*	*	*

Other compounds. The following compounds were tested and did not support growth, nor did they prevent the 'blank' growth that occurred in the complex medium: sodium dithionate (British Drug Houses Ltd., recryst. ×2), sodium formaldehyde sulphoxylate (British Drug Houses Ltd., the only available example of a compound containing the ion SO_2''), sodium benzene-sulphonate (British Drug Houses Ltd.), ammonium perdisulphate, ammonium sulphamate (Baird & Tatlock), sodium methanesulphonate (synthetic), sodium β-hydroxyethanesulphonate (synthetic), sodium ethylsulphate (synthetic) and dimethylsulphone (synthetic). For completeness, sodium sulphide was tested as a substrate for growth and was inactive, though its presence accelerated the 'blank growth', owing possibly to its strong reducing properties. Growth tests with these substances are recorded in Table 1.

Hydrogen uptake with cell suspension

Without substrate. Resting cell suspensions took up hydrogen slowly over a long period in the absence of reducible substrates; the $-Q_{H_2}$ was *c.* 15 mm^3/mg./hr. (Fig. 3). In one experiment this 'blank' hydrogen uptake persisted for 48 hr., though the quotient was by then reduced.

Sulphide was not formed during the 'blank' hydrogen uptake, which, therefore, was unlikely to be due to the reduction of intracellular stores of sulphate-like material. Also, the rate of hydrogen absorption was unaffected by 10^{-3} M sodium selenate, which could have been sufficient to prevent the reduction of about 2×10^{-2} M sodium sulphate (Postgate, 1949).

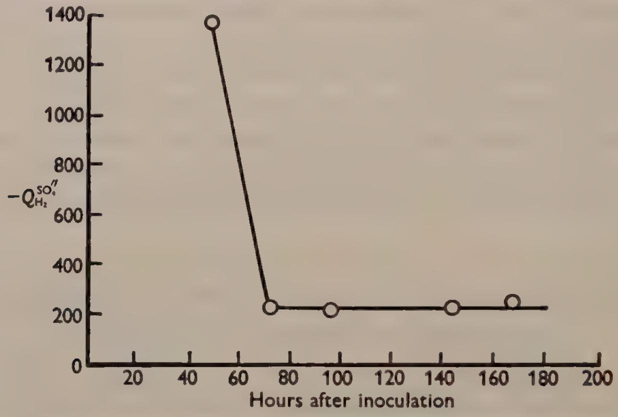

Fig. 2. Effect of the age of cultures of *D. desulphuricans* (Hildenborough) on the specific rate of sulphate reduction.

Sulphate. Resting cell suspensions in the Warburg manometer absorbed 4 mol. hydrogen/mol. sulphate, and 1 mol. sulphide was formed (Table 2, Fig. 3). This is consistent with the work of Stephenson & Stickland (1931). The specific rate of hydrogen absorption ($-Q_{H_2}^{SO_4''}$) depended on the concentration of cells present, between 0·218 and 2·18 mg. dry wt. cells/ml., and was independent of the sulphate concentration between 10^{-3} and 10^{-1} M-Na_2SO_4. Charcoal, which might have provided a greater surface for inter-action between the gas and liquid phases, did not affect the quotient, nor did ferrous ions, which remove sulphide from solution as ferrous sulphide.

The effect of age of the cells on the specific rate of hydrogen absorption is indicated in Fig. 2. Cells taken from a culture less than 48 hr. after inoculation showed an enhanced $-Q_{H_2}^{SO_4''}$; this corresponded to the period of active cell-division in the growth medium. In the stationary phase, however, the quotient remained constant for several days, so cultures in this phase of growth were used for general comparative work. The $-Q_{H_2}^{SO_4''}$ of such cells was in the region of 200 mm^3/mg./hr. for *D. desulphuricans*, strain 'Hildenborough', though values between 150 and 350 mm^3/mg./hr. have been obtained with this strain.

The greater specific rate of hydrogen absorption during growth suggests that sulphate reduction is the main energy-yielding reaction for cell mutiplication; Subba Rao & Sreenivasaya (1947) have shown that the rate of sulphate utilization by a halophil is greater during active cell-division, which is consistent with this view.

Although several substances, including lactic acid and glucose can be used as hydrogen-donors for cell growth in the absence of hydrogen, they were not utilized by resting cell suspensions when gaseous hydrogen was available. Glucose (1 %, w/v), peptone (1 %, w/v) and yeast extract (1 %, w/v) had no effect on the rate of sulphate reduction or on the amount of hydrogen absorbed/ion of sulphate. Sodium lactate (0·35 %, w/v) decreased the $-Q_{H_2}^{SO_4''}$ slightly but did not affect the total amount of hydrogen taken up.

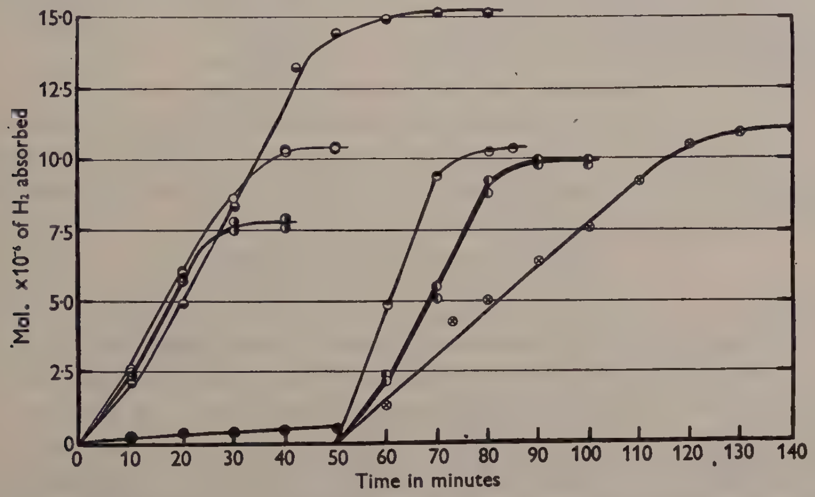

Fig. 3. Hydrogen adsorbed by resting cells of *D. desulphuricans* (Hildenborough) with various substrates.

The curves shown were taken from several experiments and their slopes are not directly comparable. $-Q_{H_2}$ values for the curve are given below. 2·5 μmol. of substrate were added in each test except in the case of sodium tetrathionate, when half this amount was used.

○ Sulphate, Na_2SO_4	210	◓ Dithionite, $Na_2S_2O_4$	220
◑ Sulphite, Na_2SO_3	210	◐ Thiosulphate, $Na_2S_2O_3$	164
◒ Metabisulphite, $K_2S_2O_5$	230	⊗ Tetrathionate, $Na_2S_4O_6$	60
● No added substrate	5·5		

Sulphite. Three mol. hydrogen were taken up/mol. Na_2SO_3 reduced (Fig. 3) and one mol. sulphide was formed (Table 2). For a given suspension, the $-Q_{H_2}^{SO_3''}$ was greatest at pH 7·0, but it was only slightly lower at pH 6·3. The $-Q_{H_2}^{SO_3''}$ was in general quantitatively similar to the $-Q_{H_2}^{SO_4''}$ for the same strain, i.e. about 200 mm³/hr./mg., though on some occasions the sulphite quotient was found to be greater than the sulphate quotient.

Thiosulphate. Four mol. hydrogen were absorbed during the reduction of one mol. Na_2SO_3 (Fig. 3) and two mol. sulphide were formed (Table 4), corresponding to the equation:

$$S_2O_3'' + 4H_2 = S'' + H_2S + 3H_2O \text{ (}or\text{ } 2HS' + 3H_2O\text{)}.$$

The optimal pH value for thiosulphate reduction was 7·0 ± 0·2, and the $-Q_{H_2}^{S_2O_3''}$ of a given suspension under optimal conditions averaged about 60 % of the $-Q_{H_2}^{SO_4''}$.

Table 4. *Sulphide formed and hydrogen absorbed by resting suspensions of* D. desulphuricans (*Hildenborough*) *with various substrates*

Substrate	Amount added (μmol.)	Hydrogen absorbed (μmol.)	Sulphide formed (μmol.)
Sulphate, Na_2SO_4	2·5	9·4 (10)	2·42 (2·5)
Sulphite, Na_2SO_3	2·5	7·3 (7·5)	2·40 (2·5)
Thiosulphate, $Na_2S_2O_3$	2·5	10·0 (10)	5·0 (5)
Tetrathionate, $Na_2S_4O_6$	1·25	11·2 (11·5)	4·82 (5)
Metabisulphite, $K_2S_2O_5$	1·5	8·9 (9)	3·0 (3)
Dithionite, $Na_2S_2O_4$	2·5	10·0 (10)	3·35 (2·5)

Theoretical figures are given in brackets.

Tetrathionate. The complete reduction of tetrathionate should theoretically take place in accordance with the equation:

$$S_4O_6'' + 9H_2 = S'' + 3H_2S + 6H_2O,$$

but the uptake of 9 mol. of hydrogen proved difficult to demonstrate. Indeed, an early report from this laboratory (Report, 1950) stated that 7 mol. of hydrogen/tetrathionate ion were absorbed. This discrepancy was probably attributable to the difficulty experienced in preparing pure specimens of sodium tetrathionate, for a 97 % pure specimen which was obtained later gave hydrogen uptakes in accord with theory: 9 mol. of hydrogen were found to be absorbed/ion of tetrathionate added, and 4 mol. of sulphide were formed (Table 4). Even with this relatively pure specimen of sodium tetrathionate, however, the total hydrogen uptakes varied more than usual and hydrogen uptakes less than theoretical were not uncommon; for example, six parallel tests with the 97 % pure specimen gave hydrogen uptakes of 8·9, 8·45, 8·1, 9·4, 8·5 and 9·0 mol. of hydrogen/tetrathionate ion. The reason for these deviations is not known. The optimal pH for the reduction of tetrathionate was pH 8·2 and the optimal $-Q_{H_2}^{S_4O_6''}$ amounted to only about 30 % of the $-Q_{H_2}^{SO_4''}$.

Dithionite. Four mol. hydrogen were taken up/ion of dithionite added, a result consistent with the view that the ion decomposes primarily to sulphate and sulphur, the first of which is reduced. However, sulphide-formation was consistently in excess of 1 mol. S″/ion S_2O_4'' required by the equation:

$$S_2O_4'' + 4H_2 = S'' + S + 4H_2O$$

though insufficient for the equation:

$$S_2O_4'' + 5H_2 = S'' + H_2S + 4H_2O$$

(see Table 4). Since freshly-prepared solutions of the specimen smelled of H_2S it is possible that the discrepancy was due to sulphide impurities. If this was the case, however, the values obtained for the hydrogen uptake must be regarded as excessive. The question was not studied further since dithionite is most unstable and very difficult to obtain pure.

Metabisulphite. Potassium metabisulphite was reduced straight-forwardly in the Warburg manometer with the uptake of 6 mol. H_2/ion S_2O_5'' and with the formation of 2 mol. sulphide (see Table 4):

$$S_2O_5''+6H_2=2HS'+5H_2O.$$

Sulphur. No hydrogen uptake in excess of the blank was detected in the Warburg manometer using 're-precipitated' or re-distilled sulphur (10^{-5} g-atom/flask). Sulphur added as a colloidal sol containing polythionates (0·0194 % $H_2S_5O_6$) showed at first a slow hydrogen uptake ($-Q_{H_2}=20$ mm³/mg./hr.) in excess of the blank and its ultra-filtrate ($-Q_{H_2}=7$ mm³/mg./hr.). To see whether this was a genuine reduction of sulphur the flasks were shaken for 16 hr. in all, by which time the $-Q_{H_2}$ in all vessels was almost zero. The total amounts of hydrogen absorbed in the flasks containing sulphur corresponded to reduction of the sulphur present, and proportionate amounts of sulphide were found (Table 5). Traces of sulphide were also found in the flasks containing the ultra-filtrate of colloidal sulphur, and a small amount of hydrogen had been absorbed, but this can reasonably be ascribed to polythionate impurities known to be present in the sulphur sol.

Table 5. *Sulphide formed and hydrogen absorbed by resting suspensions of* D. desulphuricans (*Hildenborough*) *with colloidal sulphur and its ultra-filtrate*

Substrate	Amount added (µmol.)	Hydrogen absorbed (µmol.)	Sulphide formed (µmol.)
Sodium sulphate	2·5	10·1 (10)	2·54 (2·5)
Colloidal sulphur	4·66	5·30 (4·66)	5·05 (4·66)
Ultra-filtrate	*	0·62 (0)	0·203 (0)
No addition	—	0·40 (0)	0 (0)

Theoretical figures given in brackets.

* A volume of ultra-filtrate equivalent to 4·66 µmol. of sulphur in the unfiltered sol was added.

Other compounds. The following compounds were tested and were not reduced by resting cell suspensions: sodium dithionate, sodium formaldehyde sulphoxylate, ammonium perdisulphate, ammonium sulphamate, sodium methanesulphonate, sodium β-hydroxyethanesulphonate, sodium ethylsulphate, and dimethylsulphone. In view of the growth-promoting action of cysteine (Report, 1950; Postgate, 1951), cystine (British Drug Houses Ltd.) was tested in the Warburg manometer but was not reduced.

DISCUSSION

D. desulphuricans strain 'Hildenborough' was able to utilize sulphate, thiosulphate, sulphite and tetrathionate for growth and its resting metabolism.

It was also apparently able to use dithionite and metabisulphite, but it is likely that these compounds decompose spontaneously to sulphate and sulphite respectively before use. The organism was unable to utilize dimethylsulphone or several sulphur-containing oxy-acid ions, including two aliphatic and one aromatic sulphonates.

This and other strains of *D. desulphuricans* were unable to utilize elementary sulphur purified by re-distillation, and it is unlikely that this failure to grow was due to an allotropic change in the sulphur during purification since *Th. thio-oxidans* could use it for growth. Nor is it likely that an adaptive change in the bacteria is needed for sulphur utilization, since the four strains tested in autotrophic culture were first grown with impure sulphur—a condition likely to favour such an adaptation. No sign was obtained of growth-inhibitory impurities in the purified sulphur, since the 'blank' growth and hydrogen uptake was not prevented by these preparations. It is, therefore, likely that the activity of some sulphur preparations is due to the presence of oxidation products as impurities, and this view is favoured by the fact that a purified specimen of sulphur regained its activity after prolonged standing in air. In his original work on this subject Baars (1930) used colloidal sulphur prepared by the action of excess H_2S on SO_2, but this method also yields polythionates other than dithionate, which could have been responsible for the activity observed. Baars ensured the absence of sulphate and sulphite in his preparations, but did not check them for polythionates. In the present work, a sulphur sol prepared by a similar method was active, and since its ultra-filtrate was relatively inactive, the effect of the sol was probably not due to polythionates. In Baar's work, the yields of sulphide obtained from colloidal sulphur did not depend on the amounts of sulphur added, but in this work a direct quantitative relationship, between hydrogen absorbed, sulphide formed and colloidal sulphur added was found, which constitutes further evidence for the direct reduction of colloidal sulphur.

D. desulphuricans was able to use tetrathionate in place of sulphate, but unfortunately specimens of pure pentathionate and trithionate were not available for testing. The organism did not use dithionate, a result consistent with the theoretical view that this ion is not of the same structural type as the higher polythionates (Ephraim, 1934).

The mechanism of sulphate reduction. These studies raise some interesting points concerning the mechanism of the reduction of sulphate. The main substances found to be reducible were sulphate, sulphite, thiosulphate, tetrathionate and colloidal sulphur, dithionite and metabisulphite being of secondary importance since they probably acted by spontaneous decomposition to one of the other ions. It is unlikely that sulphite, thiosulphate or tetrathionate underwent a preliminary decomposition to sulphate since the hydrogen uptakes and sulphide yields corresponded quantitatively to the reduction of the unchanged substrate: a preliminary decomposition would decrease the sulphide yield by formation of inactive sulphur, as may occur in the case of dithionite, and a preliminary oxidation to sulphate would cause considerably increased hydrogen uptakes to be found. Thus there is no doubt that these substances, as well as colloidal sulphur, are directly reduced by the organism, but it does not follow that they are necessarily intermediate in the normal reduction of sulphate. Since the sulphite ion requires three molecules of hydrogen for its reduction, as compared with four hydrogen molecules required by sulphate, the fact that the $-Q_{H_2}$ values were similar with these two compounds implies that the molar rate of sulphite utilization was greater than the molar rate of sulphate utilization by about 33 %. This suggests that sulphite is a normal intermediate in sulphate reduction, and this view is supported by the report (Postgate, 1949) that sulphite will overcome the inhibition of sulphate reduction by sodium selenate in a non-competitive manner. On the other hand, it is unlikely that thiosulphate and tetrathionate are intermediates in the normal reduction process since the specific rates at which these two compounds are reduced are less than those for sulphate and sulphite. These rates have been measured as the $-Q_{H_2}$ values for various substrates, and, whereas the $-Q_{H_2}^{SO_3''}$ and $-Q_{H_2}^{SO_4''}$ were usually similar for a given cell suspension, the $-Q_{H_2}^{S_2O_3''}$ was lower by about 40 % and the $-Q_{H_2}^{S_4O_6''}$ by about 60 %. Similarly, colloidal sulphur is probably not a normal intermediate since the $-Q_{H_2}^{S}$ amounted to only about 10 % of the $-Q_{H_2}^{SO_4''}$. The conclusion that thiosulphate and tetrathionate are not normal intermediates in sulphate-reduction is not surprising on theoretical grounds. Thiosulphate and tetrathionate are both less oxidized than sulphite, so they would presumably come at stages beyond sulphite in a scheme such as:

$$SO_4'' \longrightarrow SO_3'' \longrightarrow \ \longrightarrow S''.$$

But the scheme as written above involves only divalent ions containing one sulphur atom, and for the appearance of ions containing more sulphur atoms a loss of electrons from the system must take place, e.g.:

$$2SO_3''+3H_2=S_2O_3''+3H_2O+2e'.$$

This is a formal oxidation, but is nevertheless not an impossible reaction, and one could picture a series of oxidative polymerizations occurring, coupled with reductions, which would lead to sulphide by way of polythio-ions. However, not only would electrons be continually lost in this process, but a logical result would be the formation of free sulphur or a very complex polythio-ion. Moreover, for the stages which can be represented as:

$$\text{polythio-ion } \textit{or} \text{ sulphur} \longrightarrow \text{sulphide}$$

a considerable absorption of electrons by the system would be required as the converse of the oxidative polymerization stages.

Given suitable balance of the oxidative and reductive stages, hypotheses on these lines representing the mechanism of sulphate-reduction are not out of the question, but it is simpler to regard the normal process as:

$$SO_4'' \xrightarrow{H_2} SO_3'' \longrightarrow \left\{ \begin{array}{c} \text{unknown intermediates} \\ \text{containing 1 atom of} \\ \text{S per divalent ion} \end{array} \right\} \longrightarrow S''$$

and to regard the reduction of thiosulphate and tetrathionate as an independent process which would probably be written as:

$$S_4O_6'' \xrightarrow{H_2} S_2O_3'' \longrightarrow \left\{\begin{matrix}\text{further reductive}\\ \text{depolymerization}\end{matrix}\right\} \longrightarrow S''.$$

Even so, there is no evidence from the present work that thiosulphate is an intermediate in tetrathionate reduction, and it is possible that the two compounds are reduced by independent mechanisms.

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The Action of Normal Mouse Serum on Influenza Virus

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SUMMARY: Normal mouse serum contains a haemagglutination inhibitor and a neutralizing factor for influenza virus. The inhibitor and the neutralizing factor are heat-labile, can be destroyed by a crude filtrate of *Vibrio choleræ* and are much more active towards unadapted virus than towards mouse-adapted virus. The inhibitor enters into stable combination with the virus in the presence of Ca ions. It is adsorbed by a large amount of unadapted virus, even after the haemagglutinin of the latter has been destroyed by heating at 60° for 15 min. Three strains of freshly isolated influenza B virus tested were not significantly neutralized by normal mouse serum. By growing two strains of unadapted influenza A virus in eggs in the presence of normal mouse serum, two variants which are resistant to the neutralizing action of mouse serum were produced. It appears that this type of variation also occurs when an unadapted virus is passed in mice. The theoretical implication of this type of variation in response to a normal host component and its relationship to the mouse-pathogenicity of influenza virus are discussed.

The presence of haemagglutination inhibitor for influenza virus in normal human and animal sera has been described by various workers (Hirst, 1942, 1948; Francis, 1947; Anderson, Burnet & Stone, 1946; McCrea, 1946; Burnet & McCrea, 1946; Friedewald, Miller & Whatley, 1947). Burnet & McCrea (1946) found that normal ferret serum possessed a small inactivating power against one strain of influenza A virus (BEL) both in eggs and in mice. Smith & Westwood (1949) found that normal guinea-pig serum reduced the infectivity of PR 8 virus in mice. Typical 'Francis inhibitors', i.e. inhibitors present in normal tissues, secretions and sera resembling the inhibitor in normal human serum originally described by Francis (1947), do not neutralize influenza virus *in vivo*. Therefore, other serum factors are probably responsible for the inactivating effect observed. McCrea (1948) suggested that at least two types of normal haemagglutination inhibitors have been observed: the 'Francis' type of inhibitor and the 'non-specific inhibitor'. While the present study was in progress, Ginsberg & Horsfall (1949*a*) reported the presence of a heat-labile inhibitory and neutralizing component against Newcastle disease, influenza and mumps viruses in the serum of man, rabbit, guinea-pig and mouse. Most of their experimental work was carried out with guinea-pig serum and only laboratory-adapted strains of influenza virus (PR 8 and Lee) were employed. Their results, however, left little doubt that the guinea-pig serum inhibitor with which they dealt was in many ways similar to the normal mouse serum inhibitor reported in this paper.

The present study was initiated by the observation that unheated normal mouse serum (N.M.S.), while inhibiting to fairly high titre haemagglutination by strains of influenza A virus which had not been adapted to mice, was very much less inhibitory to the mouse-adapted strains. It was further observed that this differential inhibition *in vitro* was often accompanied by a similar differential neutralization *in vivo*. It seemed that a more thorough investigation of these phenomena might lead to a better understanding of the mechanism of mouse adaptation of influenza virus. Accordingly, studies were undertaken to investigate the properties of these normal serum factors and their mode of interaction with the virus. Experiments were also made to grow the virus in eggs in the presence of normal mouse serum and in this way two variants were produced which are resistant to neutralization by the normal serum factor. The results of these studies are described in this paper.

For the sake of clarity, throughout this paper, the term 'inhibition' or 'inhibitor' refers to haemagglutination inhibition, and the term 'neutralization' or 'neutralizing factor' refers to inactivation of infectivity *in vivo*. The term 'adaptation' or 'adapted', unless otherwise specified, always refers to mouse adaptation.

MATERIALS AND METHODS

Virus strains. Many strains of influenza virus, of both type A and type B, were used in the present investigation. As far as they are known, the origin and past history of these strains will be indicated when they are first mentioned in the text. Thus, Barratt (A/England/1947/M14) means that this strain is a type A virus isolated in England in 1947 and has had 14 mouse passages in addition to passages in eggs. Allantoic fluids for haemagglutination and inhibition tests were stored at 4°. Viruses for infectivity or neutralization tests were always stored over solid carbon dioxide.

Normal mouse serum (*N.M.S.*). Healthy mice of 'Parkes' stock of the National Institute for Medical Research, weighing 15–20 g. were used throughout. Blood was collected by putting the mice under deep anaesthesia, opening the chest and severing the aorta just above the diaphragm. Sera from 40 or more mice were pooled. With such large pools, only slight variations in inhibitory and neutralizing activities were observed between the individual pools. Mouse serum often contains a small amount of normal agglutinin against fowl red cells, which was removed by absorption before each pool was stored. The serum was then filtered through a 0·75 μ. Gradocol membrane and stored in a $-60°$ deep-freeze. Unless otherwise stated, serum was always used without prior heat inactivation.

Haemagglutination and haemagglutination-inhibition tests. The technique used has been described in detail elsewhere (Chu, Andrewes & Gledhill, 1950). Tests were carried out at room temperature with 0·5 % (v/v) fowl red cells. Eight partial agglutinating units of virus were used in the inhibition test. The virus dilution showing a partial agglutination pattern was taken as the haemagglutination titre. The highest serum dilution giving complete inhibition of agglutination was taken as the inhibition titre. Normal mouse serum in low dilutions often causes slipping of agglutinated red cells, presenting a false negative pattern which sometimes makes the placing of titre rather difficult. This difficulty was overcome when, in addition to direct reading of the pattern, the racks were held at an angle of 45° for about 30 sec. and the flow of the red cell 'button' at the bottom of the tubes observed. Unagglutinated cells should flow freely and agglutinated cells, not at all. Cells in the tube corresponding to the inhibition titre usually flow slowly but less freely than the negative control cells. All titres are expressed in terms of the initial dilution of the materials concerned.

Infectivity titration and neutralization test in eggs. The constant serum method was adopted. Equal volumes of undiluted N.M.S. and tenfold dilutions of virus in broth-saline were mixed and incubated at 4° for 1/2–1 hr. Volumes of fluid (0·2 ml.) were injected into the allantoic cavity of 9–11-day-old chick embryos, using 4 eggs/dilution. Following incubation at 35° for 3 days, some allantoic fluid was aspirated-off and tested. Presence of haemagglutinin was taken as evidence of virus multiplication. Control virus titrations were always set up at the same time, with broth-saline replacing N.M.S. The 50% infective dose (ID 50) was calculated according to Reed & Muench (1938). The number of ID 50 neutralized by the undiluted serum will be referred to as 'neutralization index' which is calculated by dividing the ID 50 for the virus mixed with serum by the ID 50 for virus control.

Passage of virus in eggs with normal mouse serum. The same technique as used for the neutralization test was followed. There was usually considerable neutralization by N.M.S. on the first passage. Fluid from one egg at the highest positive dilution was picked at random for passage, again in mixture with N.M.S. Such passages were repeated four or five times. A parallel control line was carried in eggs with broth-saline replacing N.M.S. and passed at dilutions comparable to those of the experimental line.

Infectivity titration in mice. Five 'Parkes' mice (12–15 g.) were used for each dilution. They were lightly anaesthetized with an ether-chloroform mixture and were inoculated intranasally with 0·05 ml. of virus. All mice dying within 14 days were autopsied as soon as possible; surviving animals were killed by gas and examined for lesions. Occasional mice dead from non-specific causes were excluded from the analysis of results.

EXPERIMENTAL

Haemagglutination-inhibition of influenza A strains by N.M.S.

Inhibition by N.M.S. to moderate titres seems to be characteristic of all A-prime strains isolated and maintained in eggs since 1946. Practically equal titres were obtained whether active virus or virus heated at 56° for 30 min. in citrate-borate buffer (Stone, 1949*a*) was used in the test. On the other hand, all the mouse-adapted strains including WS, PR 8 and Shope 15 were inhibited to much lower titres and even then the inhibition usually took the form of partial agglutination in several consecutive serum dilutions (Table 1). Repeated passages of an unadapted virus in the allantoic cavity of chick embryos slightly increased its inhibition by N.M.S.; thus A/Paris-1/P.L./1949 after 3 egg passages was inhibited to 1/80, the same strain after 21 passages when tested at the same time was inhibited to 1/240. In another experiment, by passing each of the two mouse-adapted strains (Paris-1/M24 and Barratt A/England/1947/M14) in eggs for 12 consecutive passages, a partial reversal to inhibition by N.M.S. (inhibition titre 1/20) was effected.

Table 1. *Haemagglutination-inhibition of influenza A strains by normal mouse serum*

Strain	Year and place of isolation	Mouse-adaptation	Inhibition titre of normal mouse serum
WS	1933, England	Adapted	<10
PR 8	1934, Puerto Rico	Adapted	<10
Barratt	1947, England	Unadapted	120
Barratt	1947, England	Adapted (M11)	<10
Williams	1947, England	Unadapted	120
Williams	1947, England	Adapted (M17)	<10
Paris-1/1949	1949, Paris	Unadapted	120
Paris-1/1949	1949, Paris	Adapted (M24)	10–20+
Roma-2/1949	1949, Rome	Unadapted	80
Roma-2/1949	1949, Rome	Adapted (M9)	10–20+

Figures under Inhibition titre in this and subsequent tables indicate the reciprocals of serum dilution. 10–20+ indicates partial inhibition at 1/10 and 1/20.

Figures in parenthesis indicate the number of mouse passages. These strains were tested with allantoic fluids from the first subculture of mouse lung virus in eggs.

Haemagglutination-inhibition of influenza virus at different levels of mouse adaptation

Strain A/Roma-2/1949 was repeatedly passaged in mice. Subcultures of mouse lungs were made in eggs after the first, third and sixth passages and the allantoic fluids tested for inhibition by N.M.S. In order to determine more quantitatively the degree of agglutination-inhibition at each serum dilution, the photoelectric densitometer method of Hirst & Pickels (1942) was adopted in this experiment. The curves shown in Fig. 1 clearly indicate that as mouse passage of the virus A/Roma-2/1949 went on, inhibition by N.M.S. was progressively decreased and after 6 passages, only partial inhibition was demonstrated. Increase in pulmonary lesions was observed after the third passage and mice began to die after the sixth passage.

Neutralization of influenza A strains

The results of inhibition and neutralization tests with the unadapted and adapted lines of Barratt and Paris-1 and with the unadapted line only of A/Nederland-1/1949 are shown in Table 2. The neutralization indices for the unadapted lines varied from $10^{2\cdot46}$ to $10^{6\cdot50}$, depending on the strain used. Since Barratt has had many egg passages before and was therefore better adapted in eggs, this may partly explain the relatively low neutralization index obtained. In general, there is no quantitative correlation between the inhibition titres and the neutralization indices when different strains are compared. Mouse-adapted viruses, however, were neither appreciably inhibited nor significantly neutralized by N.M.S.

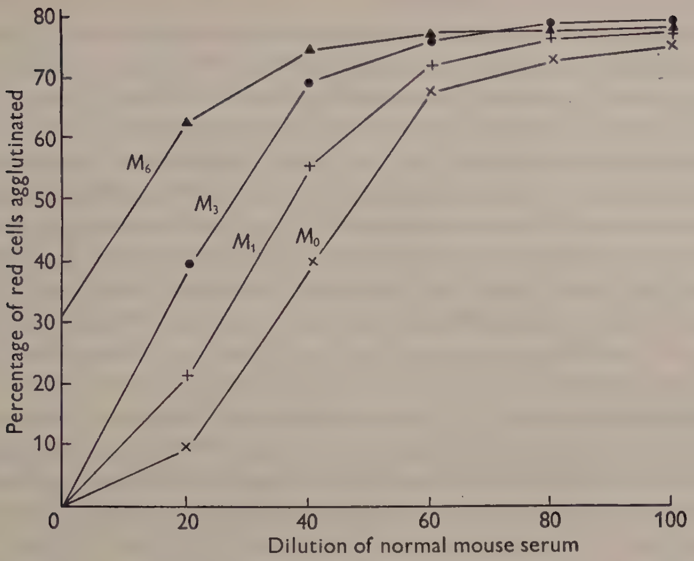

Fig. 1. Haemagglutination-inhibition by normal mouse serum of Roma-2 virus at different mouse-passage levels.

M0—virus before mouse passage.
M1, M3, M6—virus after 1, 3 or 6 mouse passages respectively and subcultured once in the allantoic cavity of chick embryos.

Effect of physical and chemical agents on N.M.S. inhibitor and neutralizing factor

(1) *Heat.* Both the inhibitory and neutralizing activities of N.M.S. were destroyed by heating the serum at 56° for 30 min. An example of such heat inactivation, tested with unadapted Barratt, is shown in Table 2. No loss of inhibitory or neutralizing activities was noticed within 6 months when a pool

Table 2. *Neutralization in eggs of unadapted and mouse-adapted strains of influenza A virus by normal mouse serum*

Normal mouse serum	Virus	Haemagglutination inhibition titre of N.M.S.	Virus titre-log ID 50		Log neutralization index
			Without N.M.S.	With N.M.S.	
Unheated	Barratt unadapted	120	8·66	6·20	2·46
Heated	Barratt unadapted	<10	8·50	>7·50	<1·00
Unheated	Barratt adapted	10–20+	7·94	7·00	0·94
Unheated	Paris-1 unadapted	80	8·50	4·23	4·27
Unheated	Paris-1 adapted	10+	8·50	>7·00	<1·50
Unheated	Nederland-1 unadapted	80	7·00	0·50	6·50

of N.M.S. was stored at −60° and tested at intervals. A pool of serum kept at 4° for one week was found to retain its inhibition titre; its neutralizing activity was not tested. A heat-labile inhibitor in rabbit serum which was inactivated by heating to 62° for 20 min. has been described by McCrea (1946). The work of Ginsberg & Horsfall (1949*a*) has already been referred to.

(2) *Adsorption with kaolin.* In previously unpublished experiments, the present author found that kaolin selectively removed the non-specific inhibitor from rabbit serum without significantly affecting the antibody titre. The same technique was applied to treat N.M.S. as follows. Kaolin (0·1 g.) and N.M.S. (0·1 ml.) were mixed and incubated in corked tubes for 30 min. at room temperature. Saline (0·9 ml.) was added and the diluted (1/10) mixture centrifuged. The clear supernatant fluid was tested for inhibition titre against the appropriate viruses.

A pool of N.M.S. originally inhibiting unadapted Barratt to 1/160 was found to be free from inhibitor after such treatment. When a pool of mouse anti-PR 8 serum was similarly treated, its titre against PR 8 was reduced from 1/240 to 1/160, whereas its titre against Barratt was reduced from 1/160 to less than 1/10.

(3) *Antagonists of Ca ions.* van der Veen & Mulder (1950) found that sodium citrate and potassium oxalate solutions used as diluent decreased the non-specific inhibition titre of ferret serum. The same effect was observed when normal saline containing 1 % of sodium citrate, potassium oxalate or sodium metaphosphate was employed as diluent in testing the N.M.S. inhibitor. Addition of 1 % calcium chloride to citrate saline restored the inhibition titre. Saline containing 1 % calcium chloride, however, did not further increase the titre of N.M.S. when an unadapted virus was used, nor was an adapted virus inhibited by N.M.S. when calcium saline was used as diluent instead of ordinary saline. A similar effect of Ca ion antagonists on the inhibition of Lee virus by normal guinea-pig serum was reported by Ginsberg & Horsfall (1949*a*) who showed in addition that a partial dissociation of the virus-inhibitor complex could be obtained with citrate saline. The N.M.S. inhibitor, therefore, resembled both the ferret and guinea-pig inhibitors in requiring Ca ions for its combination with the virus.

(4) *Sodium periodate.* The N.M.S. inhibitor was not inactivated by treatment with 0·05 M sodium periodate by the technique of Hirst (1948). The same treatment completely destroyed Francis inhibitor present in normal human serum when heated Lee virus was used as the indicator virus.

(5) *Action of* V. cholerae *filtrate and trypsin.* *V. cholerae* filtrate was prepared as described elsewhere (Chu *et al.* 1950). The receptor-destroying enzyme (R.D.E.) activity was assayed according to the method of Burnet & Stone (1947). Semi-purified R.D.E. was prepared from the filtrate by adsorption at 0° on fowl red cells and subsequent elution at 37° in a special boric acid-borate buffer containing Ca ions (Burnet & Stone, 1947). By this means, the R.D.E. activity was concentrated 2–10 times, while the slight proteinase activity found in the crude filtrate was decreased below detectable levels. Undiluted N.M.S. was treated with an equal volume of the appropriate

enzyme preparation at 37° for 18–20 hr. In order to inhibit bacterial growth streptomycin was added to give 500 units/ml. The mixtures were then adsorbed three times at 0° with a large excess of washed fowl red cells. This adsorption served to remove any residual R.D.E. which, if present in any quantity, would interfere with the subsequent inhibition or neutralization test. The successful removal of R.D.E. was ensured by adding fowl red cells to samples of mixtures and testing their agglutinability by the appropriate virus after 1 hr. incubation. An R.D.E. control was included in each experiment, treated and tested in exactly the same manner. In no case was sufficient residual R.D.E. found after the adsorption to interfere with the test. The treated serum samples were then tested for haemagglutination-inhibition and neutralization in eggs.

The results (Table 3) showed that the crude cholera filtrate destroyed the inhibitor and the neutralizing factor in N.M.S., whereas the semi-purified R.D.E. did not. It may be noted that the R.D.E. activity of the purified preparations was higher than that of the crude cholera filtrate, and since a calcium-borate buffer was used as diluent, the optimal pH and ionic conditions for R.D.E. action have been provided.

Table 3. *Effect of* V. cholerae *filtrate on the inhibitor and neutralizing factor of normal mouse serum*

		Treated N.M.S.	
Serum treated with	R.D.E. activity units/ml.	Haemagglutination inhibition titre	Log neutralization index
V. cholerae filtrate	2560	<5	1·50
V. cholerae filtrate absorbed with red cells	40	<5	nt
R.D.E. eluate	40000	40	3·50
Broth	0	40	4·23

Treated sera tested against unadapted Paris-1. nt used here and in subsequent tables denotes not tested.

Since the crude cholera filtrate used contained some proteinase, the possibility that the latter might be responsible for the inactivating effect was next investigated. Two preparations of trypsin, a semi-purified Fairchild injection trypsin, and a crystalline preparation (kindly provided by Dr A. S. McFarlane of this Institute) were tested, using the same technique as for the cholera enzyme. Under the experimental conditions, Fairchild injection trypsin, containing 100 'trypsin units'/ml. decreased the titre of N.M.S. inhibitor at least eightfold, but 0·2 % crystalline trypsin had no effect at all.

It is clear that neither R.D.E. nor trypsin itself acted on the N.M.S. inhibitor, but an unidentified active agent was present in both crude enzyme preparations.

Mechanism of virus-inhibitor reaction

The inhibition titre of N.M.S. is dependant on the amount of virus employed; within the limits of 4–16 agglutinating units, the relationship is practically linear. Outside this range, the inhibition curve deviates from a straight line, the titre being unduly high with 2 units of virus and unduly low and incomplete with 32 or more units of virus. The inhibition titre was increased by twofold if the serum-virus mixtures were incubated at room temperature for 1 hr. before the addition of red cells. Incubation beyond 1 hr. did not further increase the titre appreciably. Inhibition was very much decreased and incomplete at 4°. Approximately the same titres were obtained when guinea-pig red cells were used instead of fowl cells; with human red cells, however, the titre was decreased fourfold.

Two alternative hypotheses may be put forward to explain the mode of reaction between the virus and the inhibitor. It may be assumed that the reaction is essentially an enzymic one, and that the virus first enters into combination with the inhibitor and later destroys it. Alternatively, the virus-inhibitor reaction may be an adsorption process involving relatively firm union with no dissociation under normal conditions. If the first hypothesis is correct, one would anticipate that the virus would eventually be detected again after the inhibitor had been destroyed. This was not found experimentally. Actually, when serum and virus were mixed in such proportions that haemagglutination was just inhibited and the mixtures incubated at 37°, no reappearance of haemagglutinin could be detected in samples removed and titrated at intervals up to 24 hr. This finding in itself favoured the second view which was further tested by the following experiment.

Unit volumes of mouse-adapted or unadapted virus of equivalent haemagglutinin titre were added to equal volumes of N.M.S. and the mixtures incubated at 37° for 18 hr. The virus was then removed by filtration through Gradocol membranes of 150 mμ. A.P.D. A preliminary experiment had shown that the inhibition titre of N.M.S. was not impaired by such filtration. The resulting filtrates were titrated for inhibitor content against an appropriate virus.

When a large amount of virus (undiluted allantoic fluid with a haemagglutination titre of 1/1280) was used, the unadapted line of Barratt removed the inhibitor, whereas the mouse-adapted line of Barratt and PR8 only slightly decreased the titre. Essentially the same results were obtained when unadapted and adapted Paris-1 were tested (Table 4). When, however, the serum was treated with a smaller amount of virus (Paris-1 allantoic fluid diluted 1/16) or with undiluted allantoic fluid previously adsorbed with red cells to remove all detectable haemagglutinin, only a small decrease of inhibitor titre was effected. Unadapted Paris-1 heated at 58° for 15 min. (which destroyed its eluting power from fowl red cells), or at 60° for 15 min. (which decreased its haemagglutination titre to less than 1/10) still removed the inhibitor; even virus heated at 70° for 15 min. retained some of its activity.

It is clear that the N.M.S. inhibitor was only removed by the virus which it inhibited; a relatively large quantity of virus was required; and heating of the virus to temperatures which inactivated its other biological properties did not destroy its ability to combine with the inhibitor. These results give further support to the view that the removal of inhibitor is due to specific adsorption by the unadapted virus. Mouse-adapted virus probably has a changed surface configuration which prevents its effective combination with the inhibitor.

Table 4. *Adsorption of normal mouse serum inhibitor by unadapted and mouse-adapted influenza virus*

Adsorbing virus				Inhibition titre of adsorbed N.M.S. tested with	
Strain	Mouse-adaptation	Treatment	Haemagglutination titre	Barratt unadapted	Paris-1 unadapted
Paris-1	Unadapted	Untreated	1280	<5	<5
Paris-1	Unadapted	Heated 58° for 15 min.	640	nt	<5
Paris-1	Unadapted	Heated 60° for 15 min.	<10	nt	<5
Paris-1	Unadapted	Heated 70° for 15 min.	<1	10	5
Paris-1	Unadapted	Adsorbed with red cells	<1	30	20
Paris-1	Unadapted	Diluted 1/16	80	20	10
Paris-1	Adapted	Untreated	1280	40	15
Saline	—	—	0	60	30

nt = not tested.

Variation of inhibitor content in individual mouse sera

Since all the experiments hitherto reported were carried out with pooled sera from a large number of mice, it seemed interesting to investigate the variation of inhibition titres in the sera of individual animals. Forty healthy mice weighing 15–20 g. were bled and their sera collected and tested individually against unadapted Paris-1. The distribution of inhibition titres was 4 mice at 1/20, 6 at 1/40, 19 at 1/60, 8 at 1/80 and 3 at 1/160. This gives a characteristic median distribution curve with about 50 % of mice giving a titre of 1/60.

Haemagglutination-inhibition and neutralization of influenza B strains

Three strains of influenza B virus isolated in 1949 and 1950 were tested. They were moderately inhibited by N.M.S. in haemagglutination tests but none of them was significantly neutralized in eggs (Table 5). All three strains were initially isolated and maintained in eggs; B/London-5/1949 had had 8, and B/London-6/1950 had had 3, egg passages. Yet they were pathogenic to

Table 5. *Haemagglutination inhibition and neutralization in eggs of influenza B strains by normal mouse serum*

Strain	Haemagglutination inhibition titre of N.M.S.	Virus titre-log ID 50		Log neutralization index
		Without N.M.S.	With N.M.S.	
Budapest-1/1949	80	6·50	5·50	1·00
London-5/1949	35	6·50	5·67	0·83
London-6/1950	30	7·33	7·17	0·16

Inhibition titre against unadapted Paris-1 included as control was 1/140.
None of the influenza B strains used in this experiment has been passed in mice.

mice when allantoic fluid viruses were inoculated. The lethal dose (LD50) was characteristically low, about 10^{-1} or 10^{-2}. Dead mice showed pulmonary lesions typical of influenza infection.

Inhibitors in other animal species

Only limited data are available on inhibitors of A-prime virus strains in the serum of other animals. Normal human serum does not inhibit A-prime virus to any appreciable titre, usually less than 1/10. Other sera tested included those from sheep, ferret, rabbit, guinea-pig, hamster and cotton rat (Table 6). Hamster serum had the lowest inhibition titre. The other sera generally inhibited unadapted Barratt more than adapted Barratt, but the difference was not so marked as in the case of mouse serum. The sheep serum inhibitor was greatly diminished by heating at 56° for 30 min., and was readily destroyed by cholera filtrate.

Table 6. *Haemagglutination-inhibition of Barratt strains by unheated sera of different animal species*

	Inhibition titre tested with	
Serum	Unadapted Barratt	Adapted Barratt
Ferret	100	40
Mouse	60	<10
Rabbit	140	60
Guinea-pig	140	35
Hamster	10	<10
Cotton rat	20	<10

Of particular interest was the inhibitor present in normal ferret serum, partly because of its resemblance in some respects to the N.M.S. inhibitor, partly because of its practical importance when ferret anti-sera are used in the study of influenza virus. Like N.M.S., the inhibition titre of ferret serum was greatly decreased when citrate or oxalate saline was used as diluent (van der Veen & Mulder, 1950). The ferret serum inhibitor was only partially inactivated by heating at 56° for 30 min.; this may be due to the presence of more than one inhibitor. Treatment with cholera filtrate alone, instead of destroying the inhibitor, actually slightly enhanced its titre. After such treatment, however, the inhibitor could be completely inactivated by heating at 56° for 30 min. The mechanism of this inactivation by the combined action of cholera filtrate and heat is obscure, but since this procedure did not appear to affect to any appreciable extent the antibody of immune ferret serum, it has been applied empirically to the pre-treatment of ferret anti-sera for serological studies (van der Veen & Mulder, 1950; Chu *et al.* 1950). Treatment with purified R.D.E. followed by heating has no more inactivating effect on the ferret serum inhibitor than that achieved by heating alone.

Variants of influenza A virus produced by passage in eggs in the presence of N.M.S.

Two strains of influenza A virus, Paris-1 and Nederland-1, were employed. Both strains were isolated in 1949 and belonged to the A-prime group and were antigenically indistinguishable from each other (Chu *et al.* 1950). Paris-1 had had 1 amniotic and 25 allantoic passages before being used in the present experiment; Nederland-1 had had 2 amniotic and 2 allantoic passages. For the sake of simplicity, the parent strains will be referred to as Paris-1 Co and Ned-1 Co respectively. Viruses of the control series will be referred to as C1, C2, C3, etc., denoting virus after 1, 2 or 3 passages respectively. Similarly, viruses passed with N.M.S. will be referred to as S1, S2, S3, etc.

(1) *Paris*-1 *variant.* Data relating to the origin and characters of this variant are presented in Table 7. This strain grew well in eggs before being used in the present experiment and through 6 passages, both in the control and N.M.S. series, the haemagglutination titre did not undergo any marked change. The inhibition titres by N.M.S. also remained fairly steady in both series. Approximately equal ID50 were obtained when the two lines after the sixth passage were titrated in eggs. Both C6 and S6 were equally inhibited by ferret anti-Paris-1 serum.

Significant difference in neutralization by N.M.S. was, however, noticed between the two series even after a single passage; the neutralization index for C1, being 4·17, and for S1, being 2·00. This difference became more marked when both C6 and S6 were passed once at 10^{-8} and the subcultured fluids tested, being 4·23 and 0·83 respectively. The parent strain was quite non-pathogenic for mice, and as a rule, only very small lesions were produced, but with undiluted inoculum, an occasional mouse out of a group of five might die with complete pulmonary consolidation; such an instance was illustrated in Table 7. After 3 passages, S3 gained appreciably in mouse-virulence over C3, but it may be noted that the ID50 of the former in eggs was also tenfold higher. This increased virulence was maintained but not further enhanced in S6. In the control line, however, C6 also killed half of the mice when undiluted fluid was inoculated. Both lines were then subcultured in eggs, using 10^{-8} dilution. The allantoic fluids after the first and second subculture were titrated again in mice. The N.M.S. line consistently killed mice up to 10^{-1} dilution and occasionally at 10^{-2}, whereas the control line killed irregularly and usually less than half of the mice inoculated. It may be noted that with the exception of the third passage material, all other preparations from the two series had comparable egg infectivity titres. All the dead mice referred to had complete or nearly complete pulmonary consolidation typical of influenzal pneumonia.

Attempts were made to pass into fresh groups of mice 10 % (w/v) lung suspensions of mice which had died within 12 hr. after the inoculation of C6 or S6 materials, but only minute lesions and no deaths were produced at the two dilutions tested (undiluted and 10^{-2}). It appeared, therefore, that the pneumonia produced was of the 'non-transferable' type described by Sugg (1949).

(2) *Nederland*-1 *variant.* Data relating to the origin and characters of this variant are shown in Table 8. The parent strain grew only moderately well in the allantoic cavity of chick embryos and produced no lesions or death in mice.

Table 7. *Paris*-1 *variant produced by passage in eggs with normal mouse serum*

Passage No.	Control series						N.M.S. series					
			Mouse titre						Mouse titre			
	Neutralization index log	Egg titre log ID 50	10^0	10^{-1}	10^{-2}	10^{-3}	Neutralization index log	Egg titre log ID 50	10^0	10^{-1}	10^{-2}	10^{-3}
0	4·27	8·5	1/5	0/5	0/5	·						
1	4·17	·	·	·	·	·	2·00	·	·	·		·
2	nt	·	·	·	·	·	nt	·	·	·	·	·
3	nt	7·5	0/5	0/5	0/5	0/5	nt	8·5	5/5	4/5	1/5	0/5
4	nt	·	·	·	·	·	nt	·	·	·	·	·
5	nt	·	·	·	·	·	nt	·	·	·	·	·
6	nt	9·00	3/6	0/6	0/5	0/5	nt	8·75	7/7	5/7	1/5	0/5
6+1	4·23	9·23	2/5	3/5	0/5	0/5	<0·83	9·33	4·5	5/5	0/5	0/5
6+2		8·50	1/5	2/5	0/5	0/5		8·50	5/5	5/5	0/5	0/5

Mouse titre was expressed as No. dead/No. inoculated.
6+1, 6+2=6 experimental passages in eggs followed by one or two subcultures in eggs by passage at 10^{-8} dilution.
nt = not tested.

Table 8. *Nederland-1 variant produced by passage in eggs with normal mouse serum*

	Control series			N.M.S. series		
Passage No.	Haemagglutination titre (Inhibition titre)	Neutralization index log	Egg titre log ID 50	Haemagglutination titre (Inhibition titre)	Neutralization index log	Egg titre log ID 50
0	160 (80)	6·50	·			
1	80 60 (60) (50)	·	·	120 $\underline{160}$ (40) (40)	<5·00	·
2	200 320 (80) (100)	·	·	$\underline{8}$ $\underline{64}$ (40) (60)	1·96	·
3	240 320 240 (80) (120) (80)	>6·33	8·67	$\underline{8}$ $\underline{15}$ (40) (10)	2·21	6·63
4	320 200	·	·	$\underline{50}$ $\underline{10}$	0·75	·
5	240 320 400 (120)	·	9·00	$\underline{60}$ $\underline{50}$ (15)	1·50	7·50
5+1	640	·	·	$\underline{80}$ ·	·	·
5+2	2000	·	·	215 ·	·	·

The titres given are those of individual eggs tested. Figures in parenthesis refer to inhibition titres by N.M.S. The first eggs were used for passage and other experiments. Underlined figures refer to haemagglutination with large clumps of red cells which could not be redispersed at 37°, a rough indication that most of the virus present failed to elute.

5+1, 5+2=5 experimental passages in eggs followed by one or two subcultures in eggs by passage at 10^{-3} dilution. The titres for 5+2 represent the geometrical mean of 6 egg fluids titrations done at 4°.

Marked neutralization by N.M.S. was observed in the first passage. After 2 passages in the presence of N.M.S., three striking changes in character were observed.

Firstly, the N.M.S. line consistently gave lower haemagglutination titres relative to the control line. This relatively low haemagglutinin content in the fluid was accompanied by low ID50 figures in eggs. This character was maintained when S 5 variant was passed at 10^{-3} in the absence of N.M.S. Thus, in the second subculture, when the control and N.M.S. lines were each inoculated into 6 eggs and the fluids harvested after 72 hr. incubation were titrated at 4° (this temperature was used to keep elution to a minimum), the geometrical mean of haemagglutination titre for the control line was 1/2000 and for the N.M.S. line was 1/215.

Secondly, one out of two S 1 eggs and all subsequent eggs in the N.M.S. series yielded fluids which agglutinated fowl red cells into massive clumps. These clumps were not redispersed by tapping nor by prolonged incubation at 37°. An adsorption-elution experiment with the fifth passage material indicated that S 5 was adsorbed to fowl red cells at 4° as usual, but only 2·5 % of adsorbed virus was eluted in 2 hr. at 37° as compared with 100 % elution of C 5. This character was also maintained when S 5 was subcultured twice in eggs at 10^{-3} dilution.

Thirdly, as anticipated, increased resistance to neutralization by N.M.S. was noticeable after the first passage and was unmistakable after the second. Reduction of haemagglutination inhibition by N.M.S. was less striking, but S 5 was definitely less inhibited than the corresponding C 5. Since at the same time the variant appeared to have increased its affinity for fowl red cells, this decrease of inhibition titre may not have been entirely due to a genuine resistance to the inhibitor.

The finding of a virus which in the active state failed to elute from red cells suggested that, like heated Lee virus, it might serve as a good indicator for Francis inhibitor. This expectation was confirmed experimentally.

As inhibitor, a preparation of sheep salivary gland mucoid, obtained through the courtesy of Dr J. F. McCrea (Lister Institute of Preventive Medicine, Chelsea), was used in 1% concentration. It was found that active Ned-1 C 5 was only partially inhibited at 1/10, while active S 5 was inhibited to 1/320. Both viruses were converted into indicators, i.e. became inhibited by the mucoid inhibitor by heating at 56° for 30 min. in citrate-borate buffer (Stone, 1949*a*).

Burnet, McCrea & Stone (1946) found that different virus strains varied in the degree to which they were able to remove red cell receptors, and could be arranged accordingly into a 'receptor gradient'. A virus 'early' in the gradient removed cell receptors for the homologous virus, but still left receptors for any virus 'later' in the gradient. A virus late in the gradient removed receptors for the homologous virus and for viruses earlier in the gradient. Similarly, Stone (1949*b*) found that different virus strains could be arranged into an 'inhibitor gradient' according to the extent they were capable of destroying the inhibitor for the homologous and other strains. Isaacs & Edney (1950) demonstrated by limiting dilution technique a variant of

Melbourne virus which occurred earlier in the receptor and inhibitor gradients than the parent virus. The variation in the haemagglutination and inhibitory character of Ned-1 variant suggested the possibility that its position in the receptor and inhibitor gradients might also be altered.

The test for receptor gradient was carried out according to the technique of Burnet *et al.* (1946). Fowl red cells were incubated at 37° with undiluted Ned-1 C5 for 4 hr. when the red cells had been completely re-stabilized. The treated cells were then washed once and used for the titration of Ned-1 S5. The same agglutination titres were obtained with treated cells and control cells, indicating that C5 had removed the receptors for the homologous virus but not for S5. Unfortunately, the reverse reaction could not be tested since S5 did not elute from fowl red cells. An experiment performed by Dr A. Isaacs showed that fowl red cells treated with different concentrations of R.D.E. until they are no longer agglutinated by C5 were still agglutinated by S5.

The inhibitor-destroying test was carried out by a slight modification of the method of Burnet (1949). Samples of 1 % sheep salivary gland mucoid were incubated with 100 agglutinating units each of C5 and S5 viruses for 18 hr. at 37°. The mixtures were then heated at 65° for 30 min. and the residual inhibitory titre estimated. It was found that C5 completely destroyed the inhibitor for the heated homologous virus, but not for heated S5 or heated Lee. Destruction of inhibitor by S5 was not complete in 18 hr., suggesting a very slow enzymic action which may be related to the failure of S5 to elute from fowl red cells. However, the inhibitory titre was decreased by fourfold for heated S5, sixteenfold for heated C5 and eightfold for heated Lee. From the foregoing experiments, it may be concluded that Ned-1 variant occurred 'later' than the control line in fowl cell receptor and sheep salivary gland inhibitor gradients.

Tests for mouse-pathogenicity of Ned-1 variant were hampered by its relatively poor growth in the allantoic cavity. An attempt was made to concentrate the virus by centrifugation at 18,000 r.p.m. for 1 hr., but the deposited virus seemed to have been partly inactivated so that in spite of its higher haemagglutination titre, the ID50 for eggs was about the same as that of straight allantoic fluid. However, one experiment with S3 (−log ID50 in eggs 6·63) and another with S4 subcultured once (−log ID50 in eggs 7·00) only produced very small lesions and no deaths in mice within the tested range of undiluted to 10^{-4} dilutions.

When examined by electron microscopy after adsorption on laked fowl red cells, Ned-1 variant showed many filamentous as well as rod-like and spherical forms. The picture was indistinguishable from that of control virus. The antigenic relationship between the parent and variant lines of Ned-1 as shown by the anti-haemagglutinin test is as follows:

Serum	Virus	
	Ned-1 parent	Ned-1 variant
Anti-Ned-1 parent	280	30
Anti-Ned-1 variant	70	50

The characteristic feature was that whereas the ferret anti-serum against the parent strain inhibited the homologous virus to a titre 10 times higher than the variant, the anti-serum against the variant inhibited both to about equal titres. The two lines are antigenically closely related but not identical.

DISCUSSION

The differences between N.M.S. inhibitor and Francis inhibitor may be conveniently summarized as follows:

Normal mouse serum inhibitor	*Francis inhibitor*
Thermo-labile.	Thermo-stable
Inhibits active as well as heated virus to approximately equal titres.	Inhibits heated virus to much higher titres than active virus.
Inhibits unadapted (mouse) virus much more than mouse-adapted virus.	Inhibits both adapted and unadapted virus.
Destroyed by crude cholera filtrate, but not by purified R.D.E.	Destroyed by crude and purified R.D.E.
Not destroyed by periodate.	Destroyed by periodate.
Forms stable combination with active virus but is not destroyed by it.	Forms transient combination with active virus and is subsequently destroyed by it.
May be related to a factor which neutralizes unadapted virus in eggs.	No neutralization of virus in eggs.

The guinea-pig serum inhibitor studied by Ginsberg & Horsfall (1949*a*) closely resembles N.M.S. inhibitor. Thus, they are both heat-labile, inactive in the absence of Ca ions and enter into stable combination with the virus. These authors, however, also found their PR8 and Lee strains appreciably inhibited by unheated mouse serum. This discrepancy from the results here reported may be partly due to technical differences, but more likely is due to strain differences, as it has been shown that repeated passages of a mouse-adapted strain in eggs may partly restore its property of inhibition by N.M.S. The same authors found that their Lee strain was not neutralized in eggs by mouse serum, but both their PR8 and Lee strains were neutralized by guinea-pig serum. This finding would suggest a subtle difference between the neutralizing factors present in the two species and that a strain rendered resistant to N.M.S. by mouse-adaptation may still be neutralized by guinea-pig serum.

Both the inhibitor and neutralizing factors occur in the mouse serum; both are active against the unadapted virus and much less so against the adapted virus; both are destroyed by heating at 56° for 30 min. and by cholera filtrate. These findings strongly indicate that the two factors are closely related if not identical. On the other hand, the absence of consistent positive correlation between the inhibitor titres and neutralization indices of various strains apparently contradicts such an assumption. It may be pointed out, however, that a strict comparison of results obtained by two such different techniques can lead to fallacious conclusions. The result obtained with either technique was inherently dependant on the relative affinity between virus and inhibitor, and virus and red cells or susceptible cells lining the allantoic cavity. Furthermore, a preparation consisting of 90 % unadapted and 10 % adapted virus will behave like an unadapted virus in the haemagglutination-inhibition test and more like an adapted virus in the neutralization test. Further evidence will be needed to decide the precise relation between the inhibitory and neutralizing factors.

Inhibition and neutralization by N.M.S. appear to be characteristic of all unadapted influenza A-prime strains tested. None of the influenza B strains tested was significantly neutralized in eggs, although they were inhibited *in vitro*. It may be recalled that freshly isolated influenza B strains differ in a number of characters from the A-primes; they do not show any clear-cut O>D change (Burnet, Beveridge & Bull, 1944); they are often mouse-pathogenic to low titre without any history of mouse-adaptation and do not show filamentous forms characteristic of all freshly isolated A-prime strains (Chu, Dawson & Elford, 1949). Only a few unadapted A strains isolated in 1943 and antigenically related to PR8 have been examined by these criteria: the results obtained so far indicate that they are more akin to A-prime than to B strains.

The experimental evidence on the correlation between the mouse-pathogenicity of a strain and its susceptibility to haemagglutination-inhibition and neutralization by N.M.S. may be summarized here.

(1) All mouse-pathogenic strains, whether occurring naturally (such as the B strains) or produced by adaptation are resistant to neutralization. Their reactions to haemagglutination-inhibition were more irregular. As a rule, all freshly mouse-adapted viruses have shown significant and often striking resistance to inhibition.

(2) The converse, however, does not hold true. Ned-1 variant, for instance, which was resistant to the neutralizing factor and was significantly less inhibited by N.M.S., did not show any mouse-pathogenicity.

(3) The slightly enhanced mouse-pathogenicity of Paris-1 variant cannot be definitely correlated with the simultaneously developed resistance to neutralization by N.M.S. The facts that the control line in the course of egg passages has also gained some mouse-pathogenicity and that the pneumonia was only produced with very low virus dilutions and was of the 'non-transferable' type are obviously against the suggestion that a genuine mouse-adapted virus has been produced. Isaacs & Edney (1950) have succeeded by a limiting dilution technique in isolating a variant from Melbourne virus which appeared to show lower mouse-pathogenicity than the parent virus. It seems likely that the process of selection at high dilution inherent in the method adopted here may have quite incidentally picked out natural variants having slightly greater pathogenicity for mice.

On the foregoing evidence, it may be concluded that increased resistance to N.M.S. accompanies the process of mouse-adaptation and can be produced in eggs by passage with N.M.S. Such increased resistance may conceivably favour the multiplication of the virus in the mouse lung by overcoming a normal defence mechanism of the host, but it does not appear to be the sole or even primary factor in determining the mouse-pathogenicity of a virus. This phenomenon is nevertheless of considerable interest from the viewpoint of virus variation. Ginsberg & Horsfall (1949 *b*) reported the isolation of a variant of mumps virus resistant to the action of *Bacillus friedländer* polysaccharide by growing the virus in the presence of this chemical. Isaacs & Edney (1950) found that by passing Melbourne virus in the presence of increasing concentrations of homologous anti-serum, a mutant form was obtained which was more resistant to neutralization by anti-serum *in ovo* than the original virus. These variants, however, were only the result of special laboratory manipulations. To the author's knowledge, the N.M.S. resistant variant is the only case of variation in an animal virus which was produced in response to a normal host component and which in fact invariably occurred when the virus was adapted to this particular host. The mechanism by which the concomitant changes (such as loss of eluting power, slow enzymic activity, slower or poorer growth in the allantoic cells, shift in the gradient position and slight antigenic alteration) were brought about in Ned-1 variant is obscure. They may be independent characters selected at random. The fact that some of these characters can be readily measured by simple techniques suggests that this strain may be useful in further studies on the variation of influenza virus.

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The Antibacterial Activity of Haematin

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SUMMARY: Haematin in concentrations of 1/1,000,000–1/2,000,000 inhibited the growth of a number of bacteria most of which were aerobic sporing bacilli. Deuterohaematin and mesohaematin were also inhibitory, but not haematohaematin, urohaematin and a number of iron-free porphyrin derivatives. In a few cases whole blood was inhibitory, but generally only trypsin-digested laked red cells. The mechanism of the antibacterial activity is not understood.

This work arises from an observation by Dr R. L. Vollum that a culture of *Bacillus subtilis* appeared to autolyse very readily when grown on a blood-agar plate. It was thought that this observation might throw light on the mechanism of bacterial autolysis, but further investigation showed that autolysis was only a secondary result of the antibacterial action of haematin liberated from the blood.

These are not the first observations on the antibacterial activity of iron-porphyrins. While this work was in progress Professor Rimington pointed out that many years ago Kämmerer (1914) made the interesting observation that mesohaematin (i.e. haematin with the two vinyl groups reduced) was inhibitory to a large number of Gram-positive bacteria, including aerobic sporing bacilli, staphylococci, streptococci, pneumococci and corynebacteria. *B. anthracis* was inhibited by concentrations of 1/500,000. He believed that the antibacterial action of mesohaematin exceeded that of many antiseptics such as Karbol and Lysol, and was comparable with that of corrosive sublimate. Kämmerer did not observe any antibacterial activity of haematin, except in concentrations as high as 1/300; at 1/2,000 it tended to stimulate the growth of his test organisms.

Glass (1939) observed that haematin at a concentration of 1/100,000 was inhibitory to certain *mitis* strains of *Corynebacterium diphtheriae*.

METHODS

Bacterial growth in liquid cultures was determined by measuring the optical density (D) of the cultures with a photoelectric densitometer. D is the logarithm of the ratio of the incident light to the light transmitted through 1 cm. of the bacterial suspension. The values for D were translated into mg. dry weight organism/ml. by means of an experimentally determined standard curve. Corrections were made for the optical densities of uninoculated media. The cultures were grown in shallow layers of medium which were continuously agitated.

Proteolytic activity of bacterial cultures was determined in gelatin stabs and also by stabbing cultures into a 2 % suspension of casein in nutrient agar poured in Petri dishes. After 48 hr. incubation proteolytic colonies were surrounded by clear transparent zones in a white opaque matrix of undigested casein.

Succinic oxidase activity of washed cultures was judged by measuring the reduction time of methylene blue in the presence of succinate in Thunberg tubes. Bacterial respiration was measured in the usual way with Warburg manometers. pH values were determined electrometrically with a glass electrode.

RESULTS

Effect of blood on the growth of aerobic sporing bacilli

Petri dishes were poured with nutrient agar and after cooling a half-circle of the agar was removed and replaced by agar containing 1·5 % horse blood. The plates were then spread with an inoculum of *B. subtilis* and incubated at 37°. After 12 hr. incubation the organism had grown lightly and evenly on both halves of the plate. After 24 hr. the organisms on the blood agar were completely autolysed while the growth on the other half of the plate was profuse and normal. The organisms on this half eventually autolysed after 4–6 days. Whole blood could be replaced with washed red cells, which were more effective when laked with distilled water. Plasma had no effect. The effect with whole red cells could be obtained with only 6 out of 35 strains of *B. subtilis* that were tested, and with none of 19 strains of *B. licheniformis*. With laked red cells a few more strains of *B. subtilis* were affected.

When trypsin (25 μg. crystals/ml.) was incorporated in the laked red cell agar the effect was changed qualitatively and quantitatively. Instead of growing and then rapidly autolysing, the organism failed to grow at all. Moreover, inhibition of growth was now caused by a much lower concentration of laked red cells and affected all 35 strains of *B. subtilis* and all 19 strains of *B. licheniformis*. Control experiments showed that trypsin itself had no observable effect on these organisms. Strains of *B. subtilis* fell into at least four groups (Table 1) as far as their behaviour towards red blood cells was concerned, but they were not always consistent in their behaviour; after being stored in the cold for several months strains from group IV behaved like those from group II. The differences in behaviour of groups I and IV

Table 1. *Differences in inhibition of growth of different groups of strains of* B. subtilis *by treated and untreated red cells*

Group of *B. subtilis* strains	Growth inhibited by			
	whole red cells	red cells +trypsin	laked cells	laked cells +trypsin
I	+	+	+	+
II	−	+	−	+
III	−	−	+	+
IV	−	−	−	+

+ = inhibition

could be due to differences in their sensitivity to the inhibitory substance, or to differences in their production of proteolytic enzymes. Strains from groups I and IV were tested for their sensitivity to the inhibitory substance (which had been shown to be haematin) and they did not differ markedly, nor was there any obvious difference in their proteolytic activity against gelatin or casein. On the other hand, strains of *B. licheniformis*, which all behaved towards red cells like group IV *B. subtilis* strains, were less proteolytic towards these substrates than *B. subtilis*. It is difficult to see how differences in sensitivity or protease production could account for the fact that laked cells were more effective than trypsin-treated cells for strains of groups I and III, and less effective for group II. These differences are also apparent in Pl. 1, where in dish 1 the inhibition zone at D (laked cells) is greater than that at C (whole red cells + trypsin), but in dish 2 the reverse is true.

Some group IV strains were haemolytic, but they were inhibited only by laked red cells + trypsin, and not by haemolysed cells + trypsin. A possible explanation for this is that the cells were not haemolysed until the organisms were fully grown and therefore less sensitive to the action of haematin.

Pl. 1, dish 2, suggests that red blood cells contain a slowly diffusible stimulatory substance as well as a more rapidly diffusible inhibitory substance. There is growth on all the plugs, but those in which the inhibitory substance has been released by trypsin are surrounded by zones of inhibition. In the plugs themselves the concentration of stimulatory substance is sufficient to counteract the inhibitory substance.

It seems clear that the effect of blood on *B. subtilis* is not directly to cause it to autolyse, but to inhibit its growth. When intact red cells are used the inhibitory substance is released slowly, so that the organism has time to grow. But after a time a sufficient concentration of the inhibitory substance is released to stop the growth of the organisms, which then autolyse. It is known that *B. subtilis* autolyses readily when its growth ceases. When the inhibitory substance is released from laked cells by trypsin the organisms are inhibited immediately and they fail to grow.

The antibacterial activity of haematin

Since the inhibitory substance was liberated from red cells by proteolytic enzymes, and since concentrates of the substance were dark-coloured it was thought that it might be haem. Haemin was, therefore, prepared from laked ox blood by the method of Fischer & Orth (1937) and purified. The purified product was dissolved in dilute NaOH and its inhibitory effect tested on cultures of *B. subtilis* in potato dextrose medium (Arriagada *et al.* 1949) at pH 7·5 (where it is in the form of haematin). A concentration of 1/2,000,000 inhibited the growth of an inoculum (loopful of an overnight culture) of *B. subtilis*, but at this concentration the inhibition was only temporary and amounted to a prolongation of the lag-phase by several hours. At 1/1,000,000 it generally, but not always, prevented an inoculum from growing. Higher concentrations of haematin were needed to inhibit growing cultures (Fig. 1).

To observe the full effect of haematin it was necessary to avoid heating it in the presence of complex media. When it was autoclaved in heart broth or heart agar the concentration that was inhibitory to an inoculum of *B. subtilis* generally rose to about 1/40,000, and sometimes to 1/10,000. Autoclaving haematin in the presence of synthetic media had a much smaller effect.

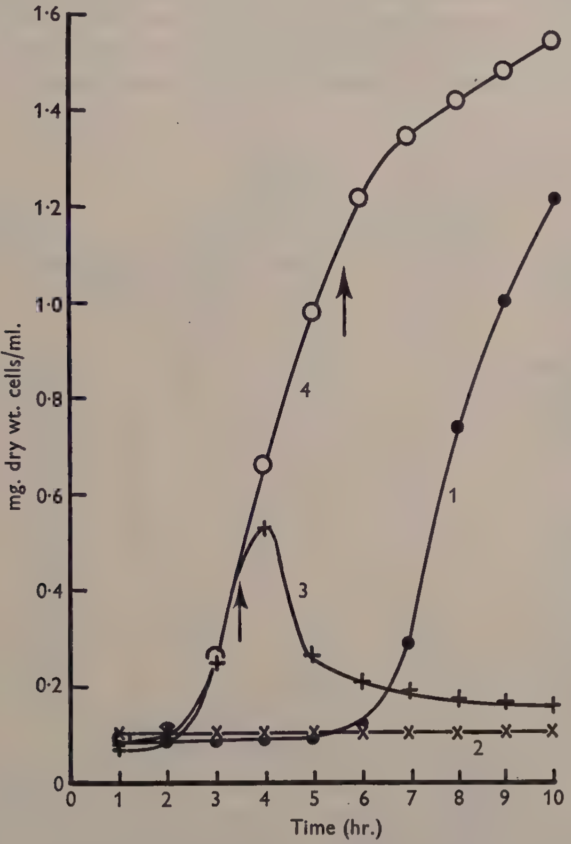

Fig. 1. Inhibition of growth of *B. subtilis* in potato glucose medium by haematin. Curve 1, 1/2,000,000 haematin added before inoculation; Curve 2, 1/1,000,000 haematin added before inoculation; Curve 3, 1/250,000 haematin added 3·5 hr. after inoculation; Curve 4, 1/250,000 haematin added 5·5 hr. after inoculation.

The effect of haematin on other aerobic sporing bacilli was tested by streaking them (3 or 4 at a time) on plates containing adjacent half-circles of plain heart agar and heart agar containing 1/30,000 haematin (added to the molten agar at 47°). The results of these experiments are shown in Table 2. In a preliminary report (van Heyningen, 1948) it was stated that *B. anthracis*, *B. laterosporus*, and *B. alvei* were not inhibited by 1/20,000 haematin, but in those experiments the haematin was autoclaved in the nutrient agar.

At a concentration of 1/20,000 haematin did not inhibit the following organisms: *Bacterium lactis aerogenes, Bact. coli, Bact. friedländeri, Chromobacterium prodigiosum, Micrococcus tetragenus, Mycobacterium phlei, Mycobact. smegmatis, Proteus vulgaris, Salmonella paratyphi* A & B, *Salm. typhi* H & O, *Sarcina lutea, Shigella flexneri, Sh. sonnei, Sh. shigae, Staphylococcus albus, Staph. aureus, Vibrio cholerae,* El Tor vibrio. At a concentration of 1/20,000 haematin completely inhibited two *mitis* strains and one *gravis* strain of *C. diphtheriae*, and partially inhibited one strain each of *C. hofmanni* and *C. xerosis*. On the other hand, it strongly stimulated the growth of two strains of starch-fermenting, gelatin-liquifying corynebacteria.

Table 2. *Inhibition of aerobic sporing bacilli by haematin* (1/30,000)

Organism	Number of strains tested	Completely inhibited	Partially inhibited	Not inhibited	Growth on control agar
B. alvei	8	8	0	0	+
B. brevis	12	7	4	1	+
B. circulans	10	7	3	0	+
B. laterosporus	3	3	0	0	+
B. licheniformis	19	19	0	0	++++
B. macerans	3	3	0	0	+
B. polymyxa	1	1	0	0	+
B. pumilus	5	5	0	0	++++
B. subtilis	35	35	0	0	++++
B. anthracis	2	0	2	0	++++
B. megatherium	7	0	7	0	++++
B. carotarum	7	0	2	5	++++
B. cereus	16	1	2	13	++++
B. firmus	1	0	0	1	++++
B. fusiformis	7	0	3	4	++++
B. mycoides	5	0	0	5	++++

The effect of a number of porphyrin and iron-porphyrin derivatives at a concentration of 1/20,000 was tested on *B. anthracis, B. brevis, B. cereus, B. licheniformis, B. megatherium* and *B. subtilis*, with the results shown in Table 3. These results suggest that mesohaematin is the most active of the compounds tested and that the presence of an iron atom in the molecule is essential for inhibitory activity. They also lead to the tentative conclusion that a non-polar group in positions 2 and 4 is necessary for inhibitory activity.

Keilin & Hartree (1947) found that deuterohaematin, haematin and haematohaematin at a concentration of 2×10^{-4} M almost completely inhibited the oxidation of succinic acid by highly active muscle preparations. The enzyme could be protected from this inhibitory effect by the prior addition of glyoxalin or denatured protein and they concluded that the inhibition was due to the formation of a co-ordination compound between the haematin iron and some groups in succinic dehydrogenase important for its activity. In view of this the effect of haematin (1/5,000) on the succinic oxidase systems of washed suspensions of *B. subtilis, B. cereus, B. licheniformis* and *B. mycoides* was tested, but no conclusive results were obtained. Inhibition of oxidase

Table 3. *Effect of porphyrins and iron-porphyrins at a concentration of* 1/20,000 *on the growth of certain aerobic sporing bacilli*

+ = complete inhibition; ± = partial inhibition; — = no inhibition.

Porphyrin derivative*	Groups in positions 2 & 4	Inhibitory to:					
		B. anthracis	*B. brevis*	*B. cereus*	*B. licheniformis*	*B. megatherium*	*B. subtilis*
deuterohaematin	$-H$	±	+	—	+	±	+
haematin	$-CH=CH_2$	±	+	—	+	±	+
mesohaematin	$-CH_2-CH_3$	+	+	±	+	±	+
haematohaematin	$-CHOH-CH_3$	—	—	—	—	—	—
urohaematin	$-CH_2-CH(COOH)_2$	—	—	—	—	—	—
protoporphyrin	$-CH=CH_2$	—	—	—	—	—	—
mesoporphyrin	$-CH_2-CH_3$	—	—	—	—	—	—
haematoporphyrin	$-CHOH-CH_3$	—	—	—	—	—	—
coproporphyrin	$-CH_2-CH_2-COOH$	—	—	—	—	—	—

* All these derivatives have $-CH_3$ groups in positions 1, 3, 5 and 8, and $-CH_2-CH_2-COOH$ groups in positions 6 and 7, excepting urohaematin which has $-CH_2-CH(COOH)_2$ groups.

activity was observed, but it was very variable, and the organisms (*B. subtilis* and *B. licheniformis*) whose growth was inhibited by haematin were no more affected than those (*B. cereus* and *B. mycoides*) whose growth was not inhibited.

The effect of haematin on the respiration of these organisms was also examined. No inhibition of respiration was observed with haematin at a concentration of 1/50,000, but at 1/5,000 inhibition was complete, whether or not the growth of the organisms was inhibited by haematin. The only difference that could be seen was that with organisms whose growth was inhibited (*B. licheniformis* and *B. subtilis*) there was a lag period of about 15 min. between the addition of the haematin and the cessation of respiration, whereas with those whose growth was not inhibited (*B. cereus* and *B. mycoides*) the inhibition was immediate (Fig. 2). Superficially, at any rate, this was the reverse of what might have been expected. In control experiments the respiration of *Bact. coli* was unaffected by 1/5,000 haematin.

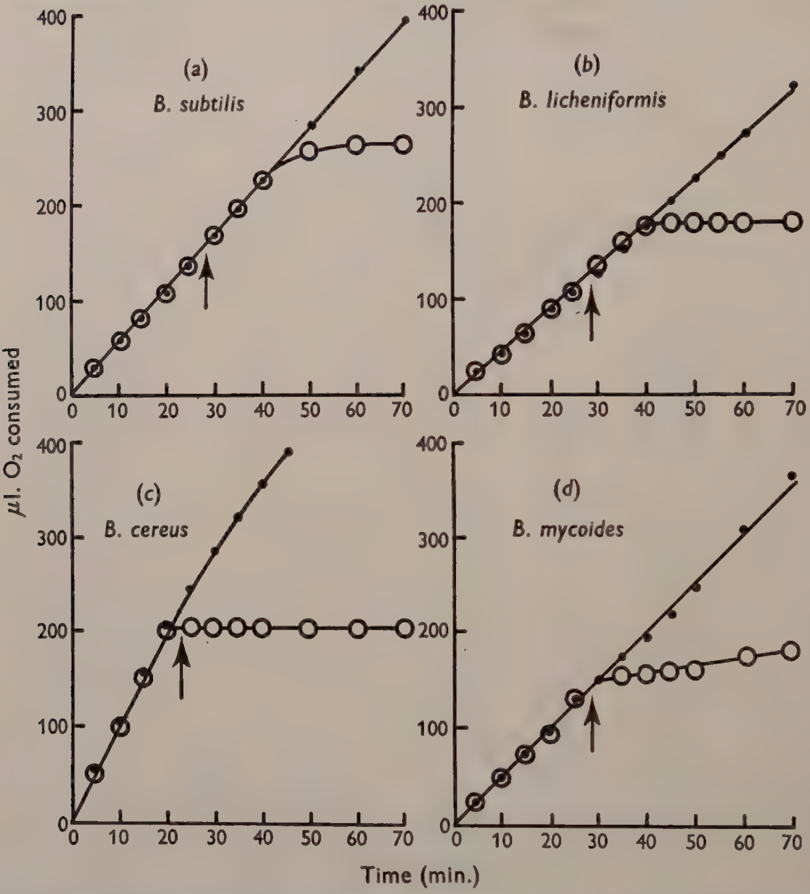

FIG. 2. Effect of haematin (1/5,000 final concentration) on respiration of organisms whose growth is inhibited by haematin (*a* & *b*) and whose growth is not inhibited by haematin (*c* & *d*). Haematin added at times indicated by arrows.

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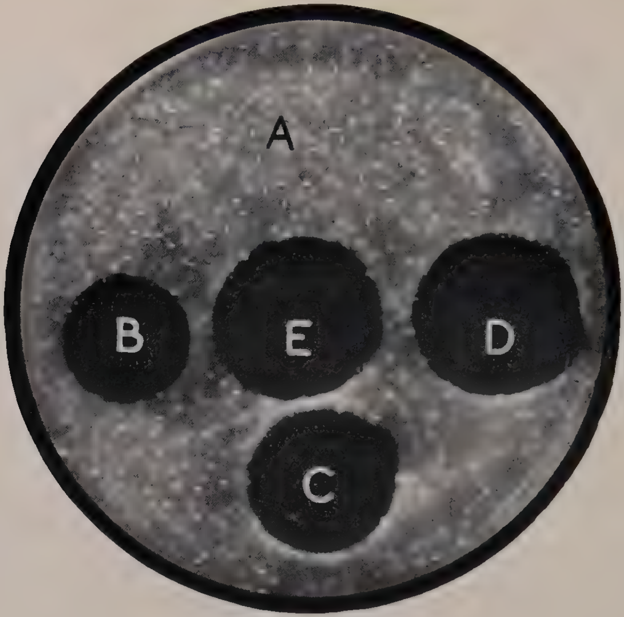
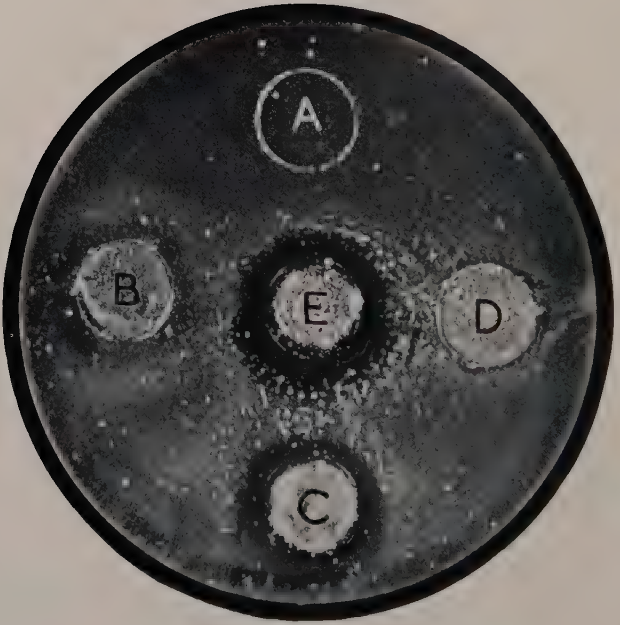


Dish 1



Dish 2

W. E. van Heyningen—The Antibacterial Activity of Haematin. Plate 1

DISCUSSION

The results reported in this paper confirm Kämmerer's interesting observations in so far as mesohaematin was found to be antibacterial and to have a greater activity than haematin. It is possible that the inactivity of haematin in Kämmerer's experience may have been due to his autoclaving this substance in his nutrient medium.

Anigstein, Whitney & Micks (1950) (see also Whitney, Anigstein & Micks, 1950) reported that tryptic digests of ticks are inhibitory to a number of species of bacteria, the aerobic sporing bacilli being the most sensitive. The antibacterial activity of extracts of ticks engorged with blood was considerably enhanced, and the inhibitory substance was shown to be associated with red blood cells. They suggest that the inhibitory substance is a peptide, but it is more likely to be haematin.

I am grateful to Dr Kathleen Crawford for strains of aerobic sporing bacilli, to Professor Rimington and Dr Joan Keilin for porphyrin derivatives, and to Miss Winifred Crawford for technical assistance.

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EXPLANATION OF PLATE

Pl. 1. Differences in inhibition of two strains of *B. subtilis* by red cells. Holes in nutrient agar plugged with agar containing: A, 25 μg. trypsin/ml.; B, 3% washed horse red cells; C, 3% red cells + 25 μg. trypsin/ml.; D, 3% laked red cells; E, 3% laked red cells × 25 μg. trypsin/ml.

Dish 1 flooded with inoculum from group I (see Table 1).

Dish 2 flooded with inoculum from group II.

The agar in both dishes contained 0·03% Neutral Red in order to simplify the photography of dishes containing red plugs. In dish 1 some of the Neutral Red has crystallized out.

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Further Observations on the Sporulation of *Chaetomium globosum*

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SUMMARY: Extracts of a strain of *Aspergillus fumigatus* stimulate fruiting of *Chaetomium globosum*, but the factor responsible is not identical with that present in jute extract. Hexose phosphates, although stimulating fruiting, do not account for the activity of the jute extract.

Buston & Basu (1948) reported the presence in extracts of jute of a substance which markedly stimulated perithecial formation of *Chaetomium globosum* Kunze, and showed that the stimulant was not identical with any of the recognized members of the vitamin B complex. Later, Basu (1951) found that the effect was in part due to the presence of calcium in the extracts. Buston & Basu (1948) also examined the influence of a number of other micro-organisms grown in association with *C. globosum*, but in no case observed any stimulation of fruiting. Later, a chance contaminant of a plate culture of *C. globosum* was found markedly to promote sporulation, the organism responsible being identified as a strain of *Aspergillus fumigatus* Fresen. From cultures of this organism, extracts were obtained having an effect similar to that produced by the jute extract. The present work was undertaken in an attempt to characterize the active substances in the extracts, and, in view of the effect of certain sugar phosphates in accelerating fruiting of *Melanospora destruens* Shear (Hawker, 1948), to examine the effect of hexose phosphates on the sporulation of *C. globosum*.

MATERIALS AND METHODS

The strain of *C. globosum* was that originally isolated by Basu from a piece of brown paper rotting in contact with moist soil, and designated by him No. 79. The basal medium contained (per litre) $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g. Glucose (British Drug Houses, Ltd., 'Analar'), 5 g./l. was the source of carbon unless otherwise stated. For solid media, agar (15 g./l.) was incorporated. Sterilization was at 10 lb./sq. in.; incubation was at 30°.

Jute extract. This was prepared from a recent sample of 'white' jute as described by Buston & Basu (1948).

Extract of A. fumigatus. As a result of many experiments to discover the cultural conditions giving the best production of the fruiting factor the following method was used: 6 l. of basal medium plus 0·5 % sucrose and 0·1 % malt extract in volumes of 500 ml. were inoculated with *A. fumigatus* and incubated at 28–30° for 8 days. Both the staled medium and an extract of the mycelium were active, but since the former still contained growth-promoting substances derived from the malt extract, mycelial extracts were preferred as a source of the active factor. The best preparations were obtained by shaking the mycelium with 0·1 N-NaOH in the presence of a little ether. The extract was neutralized with HCl before use.

Attempted chemical characterization

Attempts were made to concentrate the active material in jute extract by: precipitation with phosphotungstic acid or with barium hydroxide and ethanol; adsorption on Fuller's earth, alumina, magnesium oxide, calcium phosphate, Zeocarb, De-acidite, and activated charcoal at different pH values; extraction with ether, petroleum ether, chloroform, carbon tetrachloride, benzene, toluene, acetone, methanol, ethanol, and *n*-butanol. Partial success was obtained by adsorption on Norite at pH 2·5 followed by elution with dilute NaOH. The active substance was also partially dissolved by refluxing the dried jute extract with methanol, ethanol or 80 % acetone. However, the active substance was almost always distributed between the various fractions and no method of precipitation, adsorption or extraction gave a reasonable promise of separation. Similar experiments with Aspergillus extract likewise gave unsatisfactory results, and the only partial concentration resulted from adsorption on Norite and elution with acetone.

Further experiments showed that the jute factor was quickly inactivated by hydrolysis for 30 min. in the presence of 0·5 N-H_2SO_4 or 0·6 N-NaOH, but that it was resistant to oxidation with H_2O_2 and to bromination in aqueous solution. Tests on the dried extract showed the presence of sulphur and phosphorus (see below), and absence of halogens and, surprisingly, nitrogen.

The effect of hexose phosphates

In view of the observation of Hawker (1948) that glucose-1-phosphate, fructose-1-6-diphosphate and certain other related compounds, when present in quite low concentrations (0·05 %) in media containing glucose or sucrose, favoured the fruiting of *M. destruens*, the effect of these esters upon *C. globosum* was examined. When glucose or glucose+fructose was the source of carbon these phosphorylated compounds stimulated fruiting to an appreciable degree, but with sucrose their effect was hardly observable (Table 1). All sugar phosphates used, as well as those used by Hawker, were of biological origin, i.e. prepared enzymically from naturally-occurring materials. Thus the presence of associated growth or fruiting factors was not rigidly excluded and it could be argued that the effect of the phosphates used was due to some such impurity. To exclude this possibility a sample of glucose-1-phosphate was synthesized from bromoacetylglucose and silver phosphate (Cori, Colowick & Cori, 1937), and was found to stimulate both growth and fruiting to a marked degree (Table 1). In further experiments hexose phosphates were used in an amount equivalent to 0·5 % free hexose as the sole source of carbohydrate. Growth on glucose-1-phosphate was greater and fruiting more rapid than on the control (0·5 % glucose), but the ester was no more effective under these conditions than when present only to the extent of 10 % of the total carbohydrate. In each case fruiting occurred on the fourth or fifth day but growth was about twice as rapid on the all-ester medium. With fructose-1-6-diphosphate as sole source of carbohydrate growth was abnormal, the colonies being large and spreading but very thin and almost of the starvation type. Fruiting occurred much earlier but perithecia appeared in a ring near the edge of the colony whereas they normally form first in the centre.

Table 1. *Effect of hexose phosphates on growth and sporulation of* C. globosum *on agar media*

Sugar in medium	Phosphate added	Time of observation (days)				
		2	3	4	6	10
		Colony diameter (mm.)				
Glucose (0·25 %) + Fructose (0·25 %)	G-1-P (0·05 %)	14	19	21*	28	37
,,	F-1-6-P (0·05 %)	14·5	21	23*	27·5	36
,,	(none)	6	7·5	9·5	11·5	14·5*
Glucose (0·5 %)	F-1-6-P (0·05 %)	13	15·5*	18	20	—
,,	G-1-P (synth., 0·05 %)	15	—	25*†	—	—
Sucrose (0·5 %)	G-1-P (0·05 %)	14	23	31	39*	—
,,	F-1-6-P (0·05 %)	10	22	32	47*	—
,,	(none)	10	17	20	24*	—

* First appearance of perithecia.
† Day 5.
(G-1-P = glucose-1-phosphate; F-1-6-P = Fructose-1-6-diphosphate.)
(Colony diameter recorded is mean of not less than four measurements.)

Comparison of jute extract, aspergillus extract and hexose phosphates

Since hexose phosphates possessed some activity it seemed desirable to ascertain whether the activity of the jute and the aspergillus extracts was due to the presence of such esters. Analyses of jute extract showed that the amount used contributed 0·15 mg. total P/100 ml. final medium; of this 0·08 mg. was inorganic and 0·04 mg. was 'acid-labile' (i.e. hydrolysed by N-HCl at 100°, and considered to include glucose-1-phosphate and fructose-1-6-diphosphate). From these results it was evident that the amount of ester phosphate derived from the jute extract was very small indeed. As a further test, jute and aspergillus extracts were hydrolysed under conditions known to destroy hexose phosphates, and the hydrolysates were tested for growth and fruiting activity. The results (Table 2) showed that jute extract after hydrolysis no longer stimulated fruiting, but that the aspergillus extract was much more resistant.

Table 2. *Effect of hydrolysis on the activity of jute and aspergillus extracts, as measured by colony diameter of* C. globosum *on agar media* + 0·5 % *glucose*

Method of hydrolysis	Time of observation (days) 2	3	6	14
	Colony diameter (mm.)			
Jute extract				
N-HCl; 10 min.	14·5	—	37	>50
N-HCl; 30 min.	14	—	34·5	47
0·65 N-NaOH; 30 min.	13	—	43	>50
unhydrolysed	20	37*	—	—
Aspergillus extract				
N-HCl; 30 min.	15	—	27·5*	—
0·65 N-NaOH; 30 min.	9	—	14·5	22*
unhydrolysed	14·5	—	35*	—

* First appearance of perithecia.

The activities of fructose diphosphate and of the two extracts were also compared by the cup method. Several plates of the medium plus 0·5 % glucose and 2 % agar were made and inoculated with a spore suspension of *C. globosum*. After incubation at 25° for 5 days a cup was cut in each plate; sterile solutions of 1 % fructose diphosphate, jute extract and aspergillus extract were placed in different cups. After 2 days at 25° perithecia were observed in the colonies surrounding the cups of jute extract; after 4 to 5 days there was vigorous fruiting round the jute extract cups, rather less round the aspergillus extract and none round the fructose diphosphate.

Note on the specific activity of sucrose

Hawker & Chaudhuri (1946) drew attention to the fact that sucrose seems more favourable than any other simple sugar to the rapid fruiting of *M. destruens*, probably because the rate of inversion in this case is sufficient to maintain an optimum level of hexose sugar. In the present work it has been noted that the favourable effect of glucose-1-phosphate is suppressed when sucrose forms the main carbohydrate supply. Since sucrose is readily obtained

Table 3. *Effect of various samples of sucrose on growth and sporulation of* C. globosum *on agar media containing* 2 % *sucrose*

Sugar sample	Time of observation (days) 2	3	4	5
	Colony diameter (mm.)			
White, beet	13·5	18	19*	20
Demerara, cane	18	29	34·5*	37
Barbados, cane	19·5	28	31*	32

* First appearance of perithecia.

commercially in various degrees of refinement it seemed possible that tests on different grades of the sugar might indicate whether the activity could be ascribed to sucrose itself or to some contaminant. Refined white beet sugar, Demerara sugar, and moist brown (Barbados) sugar were used. The results (Table 3) showed that the activity of sucrose is indeed an intrinsic property of the sugar since fruiting occurred simultaneously and equally with all types. The cane sugars, however, evidently were contaminated with additional growth factors.

DISCUSSION

The prolonged attempts to separate or even to concentrate the factors present in jute extract and in *A. fumigatus* have led to little information concerning their chemical nature. Both are comparatively heat-stable in neutral solution but rapidly hydrolysed by alkali. The fact that neither is precipitated by phosphotungstic acid seems to rule out a basic substance and in fact the preferential extraction of the aspergillus factor with weak alkali indicates acidic properties. The absence of nitrogen from jute extract excludes many types of substance. The two extracts differ notably in the effect of acid hydrolysis which rapidly destroys the jute factor while under similar conditions aspergillus extract is but little affected. Differences in solubility were also noted, and it seems, therefore, that more than one substance is capable of producing the fruiting response in *C. globosum*. Basu (1947) suggested that during normal development there is a slow production of some compound within the organism which, on reaching a critical concentration, initiates fruiting. If the jute and the aspergillus factors are different the latter seems more likely to resemble the supposed intrinsic factor of *C. globosum*. Indeed, it might be considered remarkable if the jute plant produced a substance identical with that present in *C. globosum* unless the supposed substance were one of wide biochemical significance, playing a part in the metabolism of both micro-organisms and higher plants. Such substances are known and notable among them are the group of phosphorylated compounds derived from hexoses, two at least of which have been shown to assist fruiting of *C. globosum*. The comparative stability of the aspergillus factor towards acid hydrolysis shows that it is not one of the ordinary sugar phosphates (with the possible exception of the rather more stable glucose-6-phosphate), but in the case of the jute factor the behaviour on hydrolysis does not exclude the possibility that a sugar phosphate may be mainly responsible for its activity. The total amount of phosphorus introduced by the jute extract was 0·15 mg./100 ml. medium, and of this 0·04 mg. was acid-labile organic phosphorus (glucose-1-phosphate and fructose-1-6-diphosphate). Calculated as glucose-1-phosphate, this represents approximately 0·35 mg. ester/100 ml. medium; since this is so much less than the amount (50 mg./100 ml.) found to give a good response, it seems unlikely that the effect of the jute extract can be ascribed entirely to the presence of hexose phosphates.

Basu (1950) found that calcium in a limited range of concentrations (*c.* 10 p.p.m.) reproduces to some extent the effect of jute extract, but although we agree that the ash of jute does stimulate fruiting, the susceptibility of the extract to acid hydrolysis does not support the view that the calcium ion as such is responsible for its activity. The conclusion is reached that if calcium plays a part in the fruiting mechanism, and that if, as seems likely, organic phosphates are involved, neither can account entirely for the properties of extracts of jute and of *A. fumigatus*.

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A Study of the Role of Inositol in the Nutrition of *Nematospora gossypii* and *Saccharomyces carlsbergensis*

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SUMMARY: A study has been made of the uptake of inositol from liquid media by *Nematospora gossypii* and *Saccharomyces carlsbergensis* 4228. All the available inositol was rapidly removed from the medium so long as growth continued. Small amounts were recovered from the organisms by water extraction, and up to 75% from the yeast by acid hydrolysis. This bound inositol in the yeast possessed properties resembling those of phytin. On media deficient in inositol, cell division was incomplete. No influence of inositol on the respiratory system of *S. carlsbergensis* could be detected. γ-Hexachloro*cyclo*hexane inhibited growth and respiration of intact yeast cells, but not respiration of dried yeast preparations; its effect was not reversed by inositol. Streptidine, strepturea and streptamine neither inhibited growth nor could replace inositol as a growth factor.

Although *meso*-inositol was the first member of the 'bios' complex to be identified (Eastcott, 1928) its function in metabolism remains uncertain. That it takes part in some enzyme system was postulated by Slade (1945), who suggested that the toxic action of γ-hexachloro*cyclo*hexane towards some insects is due to antagonism towards *meso*-inositol, to which it was thought to be structurally analogous. Support for this theory was provided by the work of Kirkwood & Phillips (1946) who found that inhibition of growth of a strain of yeast by γ-hexachloro*cyclo*hexane was overcome by *m*-inositol (see also Buston, Jacobs & Goldstein, 1946). Lane & Williams (1948) stated that highly purified pancreatic amylase contains an exceptionally high proportion of *meso*-inositol, and that its activity is decreased by γ-hexachloro*cyclo*hexane and partially restored by *meso*-inositol; these observations have, however, been disputed by Fischer & Bernfeld (1949). The present work was designed to examine more closely the part played by *meso*-inositol (in the sequel referred to as inositol) in the growth and metabolism of certain micro-organisms.

EXPERIMENTAL

Organisms and culture methods

The organism most frequently used was a strain (4228) of *Saccharoymces carlsbergensis* known to require inositol as a nutrient. Stock cultures were maintained on malt extract agar containing 2% (w/v) of glucose. For the preparation of standard inocula, a transfer from the stock culture was grown for 24 hr. at 30° in 10 ml. of glucose malt extract broth; the cells were centrifuged and resuspended in 0·9% sterile saline to give a faintly opalescent suspension (*c.* 230,000 cells/ml.). In other experiments, *Nematospora* (*Ashbya*) *gossypii* was used, the technique being that of Buston & Pramanik (1931) except that in the medium the lentil extract supplement was replaced by biotin and aneurin (2 μg. and 1 mg. respectively/100 ml. medium).

Assay of inositol

Chemical methods for the determination of inositol are laborious and lack sensitivity, and a microbiological assay method was, therefore, sought. It had been suggested by Hopkins & Pennington (1947) that *S. carlsbergensis* 4228, employed by Atkin, Schultz, Williams, & Frey (1943) for the assay of pyridoxin, might be suitable for the assay of inositol. This was found to be the case, the method finally adopted being as follows. Five ml. of the basal medium of Hopkins & Pennington, but omitting inositol, were placed in each of a number of 50 ml. conical flasks, the solution containing inositol was added and the volume made up to 9 ml. with distilled water. After sterilization (10 lb./sq. in., 10 min.) each flask was inoculated with 1 ml. of the standard yeast suspension and incubated at 30° for 10 hr. with constant shaking. The flasks were then placed in the refrigerator for 15 min. and 1 ml. of 40 % formaldehyde solution was added to each. The cultures were then centrifuged and the cells resuspended in 10 ml. of 0·9 % saline. The amount of growth was estimated turbidimetrically by means of a Spekker absorptiometer. Satisfactory results were obtained when the amount of inositol ranged from 0 to 10 μg./10 ml., the latter figure representing the upper limit of accuracy. In some experiments it was found preferable to assess the growth by centrifuging the culture under standard conditions in a calibrated centrifuge tube, This was done because the yeast cells failed to divide normally, but remained in aggregates, thus rendering turbidimetric measurements inaccurate; instances are noted below.

Uptake of inositol by growing organisms

N. gossypii. The organism was grown on 25 ml. portions of liquid medium for periods varying from 6 to 17 days at 30°. Inositol was added to the medium in concentrations up to 6 μg./ml. At the end of the growth period mycelium was removed on sintered-glass filters; the filtrates were evaporated and assayed for residual inositol. It was found that after 8 days nearly maximum growth had taken place in the presence of only 0·4 μg./ml. inositol, although when 15 times this amount was available the greater part was still taken up; the dry weight of mycelium only increased by about one-third, however. Table 1 gives some typical results.

Table 1. *Removal of inositol from the medium by* N. gossypii

Time of observation (days)			Time of observation (days)	
0	8	17	8	17
Inositol in medium (μg./25 ml.)			Dry wt. of mycelium (mg.)	
10	0	0	27	15
50	0	0	28	25
100	0	0	32	30
150	17	12	35	28

S. carlsbergensis 4228. The use of this organism has several practical advantages over *N. gossypii*, and it was preferred for more extensive experiments. In general, 5 ml. of basal medium were placed in each of twenty 50 ml. conical flasks together with 5 ml. of inositol solution; after sterilization each flask was inoculated with 1 ml. of the standard yeast suspension and incubated with continual shaking for periods up to 68 hr. at 30°. The contents of the flasks were then centrifuged and suitable amounts of the supernatant liquids assayed for inositol. The residues in the centrifuge tubes were resuspended in 11 ml. 0·9 % saline with the addition of 1 ml. 40 % formalin, and the amount of growth was estimated by comparison with standard yeast suspensions. From these experiments (see Fig. 1) it appeared that within wide limits all the inositol was removed from the medium. With very high concentrations of inositol, uptake continued until growth ceased (owing to exhaustion of some other nutrient) when uptake ceased also. As with *N. gossypii* the final amounts of growth, even where there was a marked deficiency of inositol in the medium, were very similar when sufficient time was allowed. It was subsequently found that even on media without inositol growth occurred slowly and eventually approached the maximum, whilst inositol in a bound form appeared in the organism. Thus it seems that *S. carlsbergensis* can, to a restricted degree, synthesize inositol though not sufficiently rapidly for normal healthy growth. As appears from Fig. 1 the difference to be observed at different inositol concentrations is in the rate rather than in the total amount of growth. For the concentrations used, the rate of growth was constant and practically the same during the first 18 hr. for all cultures except that in which there was no inositol. When the initial supply of inositol became exhausted the rate of growth fell off fairly sharply and became almost the same as that for the culture containing no inositol. This change of growth rate was observed in all cases providing that other nutrients were present in adequate amounts. Under conditions where growth could not take place no uptake of inositol from the medium was ever observed.

The fate of inositol

N. gossypii was grown on a medium containing adequate inositol, the mycelium collected, dried and extracted by refluxing with water or acid. After appropriate adjustments of pH and volume, the extracts were assayed for inositol. The results (Table 2) showed that under these conditions up to about 25 % of the inositol removed from the medium could be recovered and that this amount at least is converted into a form from which inositol is liberated by acid hydrolysis.

S. carlsbergensis was grown on media containing 9 μg. inositol/ml. for periods up to 48 hr. After centrifuging, the supernatant liquid was assayed for inositol. The washed cells were dried at 98° to constant weight and then extracted in various ways, in some cases with the addition of known amounts of inositol as a check on recovery. Assays were carried out on the extracts after adjusting to pH 4·5. Typical results are given in Table 3.

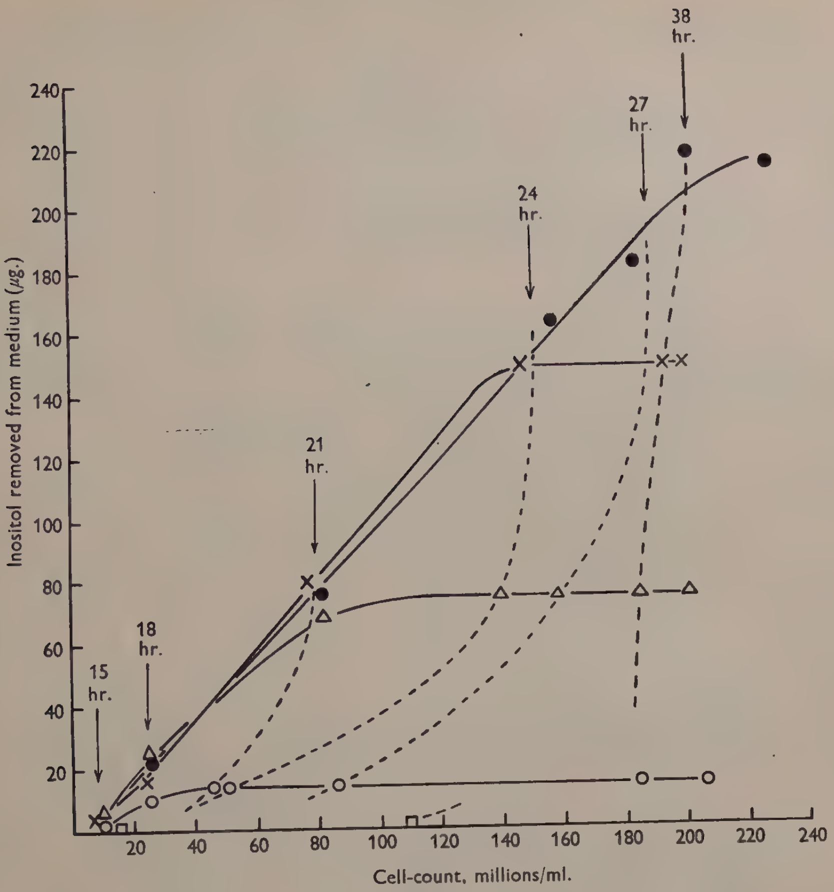

Fig. 1. Relation between uptake of inositol and growth of *S. carlsbergensis*.

Inositol initially present in the medium: —●— 300 µg.; —×— 150 µg.; —△— 75 µg.; —○— 15 µg.; —□— 0 g. Time of incubation (hr., 30°) shown above the curves.

Table 2. *Recovery of inositol from mycelium of* N. gossypii

Organism grown for 6 days at 30° on 100 ml. medium containing (*a*) 1·12 mg., (*b*) 0·42 mg. inositol.

Inositol removed from the medium (*a*) 0·98 mg., (*b*) 0·37 mg.

Method of extraction of mycelium	Inositol recovered (% of uptake) (*a*)	(*b*)
Refluxing with water, 1 hr.	12	5
Refluxing with 5N-HCl, 1 hr.	—	27·5

As noted above, slow growth of the yeast occurs even on media free from inositol. In one experiment for instance, the yeast was grown for 48 hr. on 200 ml. of an inositol-free medium. Aliquot portions (1/5) of the yeast produced were extracted with (*a*) boiling water for 20 hr., (*b*) 5 N-HCl at 100° for 20 hr. The extracts contained respectively 11 μg. and 180 μg. of inositol, showing that the yeast thus grown has a limited capacity to synthesize inositol which appeared largely in the form of a water-insoluble, acid-extractable compound.

Table 3. *Recovery of inositol from* S. carlsbergensis

(i) Organism grown for 48 hr. at 30° on 120 ml. of medium, containing 4·32 mg. inositol. Inositol removed from the medium, 3·375 mg.

Method of extraction	Inositol recovered (% of uptake)
Refluxing with water, 6 hr.	7·5
Autoclaving with water, 15 lb./sq. in., 1 hr.	3·8
Refluxing with 2N-HCl, 6 hr.	31
Autoclaving with 2N-HCl, 15 lb./sq. in., 1 hr.	29
Refluxing with 2N-NaOH, 6 hr.	21
Autoclaving with 2N-NaOH, 15 lb./sq. in., 1 hr.	16

(ii) Organism grown for 27 hr. at 30° on 120 ml. medium, containing (*a*) 4·32 mg., (*b*) 1·08 mg. inositol. Inositol removed from medium, (*a*) 1·45 mg., (*b*) 0·49 mg.

	Inositol recovered (% of uptake)	
Method of extraction	(a)	(b)
Refluxing with 5N-HCl, 20 hr.	76	50

Inositol is widely distributed in nature in the form of phytin, which is hydrolysed with difficulty (Bartow & Walker, 1938). Since it appeared possible that the bound form in which inositol is present in the organisms studied might in fact be phytin, this was estimated in the yeast by the method of Averill & King (1926). In an experiment in which 8·7 mg. of inositol had been removed from the medium by the organism, 6·6 mg. were estimated as present in the precipitated iron-phytin complex. This suggested that much of the bound inositol was indeed present as phytin, but as the method of estimation may lack specificity actual isolation of phytin is desirable.

Abnormal growth of S. carlsbergensis *lacking inositol*

In media deficient in inositol the cultures were abnormal in that the cells failed to divide completely, remaining in aggregates of up to 50 cells. Detergents had no effect upon these aggregates and it seemed possible that their formation was due to a failure in the last stages of cell division. Incubation with shaking at 30° for 48 hr. in saline solution containing up to 100 μg. inositol/ml. was without observable effect upon these aggregates.

Abnormalities in the appearance of certain micro-organisms lacking inositol have been noticed by other workers; thus Rao, Mistry & Sreenivasaya (1946) found that toddy yeast became spongy, and Beadle (1944) found that the mycelium of a *Neurospora* tended to stick to the culture flask.

The action of certain compounds related to inositol

Since the observation of Woods (1940) that the bacteriostatic action of sulphanilamide is due to antagonism with the structurally related bacterial growth factor *p*-aminobenzoic acid, many cases of similar antagonism between growth substances and structural analogues have been recorded. The discovery by Peck *et al.* (1946) that streptidine, which forms part of the streptomycin molecule, is a derivative of inositol suggested that the antibiotic action of streptomycin might arise in a similar manner. Streptidine, strepturea and streptamine were prepared from a sample of streptomycin by the method of Fried, Boyack & Wintersteiner (1946) and the effect of these substances on the growth of *S. carlsbergensis* was tested to see whether any of them would antagonize or could replace inositol; γ-hexachloro*cyclo*hexane was similarly tested. The yeast was grown for 18 hr. on the usual medium with the addition of inositol and the other compounds tested, in various combinations and in concentrations ranging from 0·00001 M to 0·0015 M. γ-Hexachloro*cyclo*hexane, being sparingly soluble, was added in strong ethanol solution, a test having shown that the presence of ethanol in similar concentration did not affect yeast growth. The results are expressed (Table 4) as the decrease of the reading on a Spekker turbidimeter due to the presence of the cells (i.e. the Spekker reading for the culture subtracted from the reading for the clear medium). This gives a measure in arbitrary units of the light absorption due to the presence of yeast cells, and varies with the number of cells present, although not directly proportional thereto. This experiment showed that, of the compounds tested, the only one possessing any activity was γ-hexachloro*cyclo*hexane which caused a definite inhibition of growth. This inhibition, however, was not reversed by inositol even when the latter was present in a concentration 35 times as great as that needed for maximum growth.

Thus it seemed that the inhibition was not of the type in which the analogue reversibly competes with the growth factor in an enzyme system. Further evidence for this rests in the fact that the abnormal type of growth typical of inositol deficiency described above was not seen in cultures inhibited by γ-hexachloro*cyclo*hexane. Streptidine, strepturea and streptamine had no growth-promoting activity.

Inositol and the respiration of certain yeasts

The effect of inositol on suitably respiring yeast cultures (*S. carlsbergensis* and a commercial lager yeast) was examined, but no significant differences in the rate of oxygen uptake could be detected. The experiments were carried out in the presence and absence of glucose and of phosphate; in some cases the yeast had been grown on a medium rich in inositol and in other cases on an

Table 4. *Effect of compounds related to inositol on growth of* S. carlsbergensis 4228

Organism grown for 18 hr. at 30°.

	Concentration of inositol in medium			
	0	0·00001 M	0·0001 M	0·0005 M
Compound tested (M concentrations)	Decrease in Spekker reading			
Streptidine,				
0·000016	—	1·23	1·33	1·33
0·0015	0·14	1·29	—	1·28
Strepturea,				
0·000016	—	1·27	1·35	1·37
0·0015	0·17	1·29	—	1·31
Streptamine,				
0·000016	—	1·23	1·34	1·27
0·0015	0·16	1·30	—	1·32
γ-hexachloro*cyclo*hexane,				
0·000016	—	0·14	0·15	0·14
(no addition)	0·16	1·25	1·32	1·33

Decrease in Spekker reading of 1·30 corresponds to approximately 25,000,000 cells/ml. under the conditions of the experiment.

inositol-free medium. These negative results do not necessarily mean that inositol is not concerned in respiration, since the yeasts even when grown in an inositol-free medium contained inositol, and cells take up inositol only while growing.

Further experiments showed that the oxygen uptake of *S. carlsbergensis* was inhibited in the presence of γ-hexachloro*cyclo*hexane; for example, in one experiment the oxygen uptake was 120 μl. O_2/90 min. in the presence of γ-hexachloro*cyclo*hexane (0·000016 M), compared with 160 μl. in a control experiment. The inhibition was not reversed by the addition of comparable amounts of inositol to the medium.

Extensive tests were also made with acetone-dried yeast preparations. With these, γ-hexachloro*cyclo*hexane exerted no inhibitory effect nor did inositol influence the rate of oxygen uptake. It appeared, therefore, that the inhibition produced by γ-hexachloro*cyclo*hexane with the intact cells was connected only indirectly with the respiratory enzymes. Finally, experiments using the Thunberg methylene blue technique failed to demonstrate the presence of an inositol dehydrogenase in *S. carlsbergensis* 4228.

DISCUSSION

Although the importance of *meso*-inositol in nutrition has been recognized for many years, little progress has been made towards the elucidation of its mode of action. Evidence that it is concerned in some enzyme system would be provided if the theory of Slade (1945) concerning the toxicity of γ-hexachloro*cyclo*hexane were proved, but observations by various workers on this subject are contradictory. (See Fromageot & Confino, 1948; Chaix, Lacroix &

Fromageot, 1948; Thorp & de Meillon, 1947). Only for pancreatic amylase (Lane & Williams, 1948) has any direct connexion been reported between inositol and a particular enzyme system. If an amylase is present in *S. carlsbergensis* it is likely to account only for a small proportion of the dry weight of the organism, whereas if the whole of the inositol recoverable from the yeast were present in the form of an amylase of a constitution similar to that studied by Lane & Williams it would constitute almost the whole of the dry material of the organism. In general, purified enzymes have not been found to contain significant amounts of inositol; this has been noted by Williams, Schlenk & Eppright (1944) for a number of enzymes, and in this laboratory for a purified potato phosphorylase prepared according to Bourne, Macey & Peat (1945). This last preparation also was unaffected by γ-hexachloro*cyclo*hexane.

Two facts seem to differentiate inositol from other members of the vitamin B group: (1) the relatively large amount required, i.e. 10 to 1000 times as much as for other B vitamins; (2) the chemically inert nature of the inositol molecule. These facts make it less likely that its principal function is that of an active component of an enzyme system. The attempts made in the present work to detect its influence on respiration, all of which yielded negative results, provide evidence supporting this view; Hopkins & Pennington (1950) suggest that it is improbable that inositol forms part of a simple coenzyme in alcoholic fermentation.

Available evidence favours the view that inositol may be used in the formation of an actual building unit in the structure of the cell. Both *S. carlsbergensis* and *N. gossypii* were found to absorb considerable amounts of inositol during growth, in a quantity proportional to the amount of growth of the organism; when the external supply of inositol was exhausted there was an immediate slowing of growth rate comparable with that on an inositol-free medium. The abnormal type of growth noticed in deficiency of inositol is also understandable if the primary function of the substance is as a necessary structural unit rather than as an enzyme constituent. Up to 75 % of the inositol absorbed during growth can be recovered by acid hydrolysis and seems to be present in a form resembling phytin. The amount of inositol recoverable both by water and acid extraction is greater when the yeast is grown on media relatively rich in inositol. Some organisms have been reported to oxidize inositol to the corresponding mono- or di-keto derivatives (Carter *et al.* 1948), but these substances have not been detected during this work.

The slow synthesis of inositol by *S. carlsbergensis* 4228 makes it important that the incubation period in assays with this organism should not be too long; since, however, there is practically no growth in the absence of inositol during the first 18 hr., the assay method using this period may be considered satisfactory.

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Mutations Affecting the Nutritional Requirements of *Aerobacter aerogenes* Induced by Irradiation of Dried Cells

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SUMMARY: By irradiating with X-rays *Aerobacter aerogenes* (AlOC) in the dry state, mutants have been obtained differing from the parent strain in specific growth-factor requirements. About half of the mutants differ from the parent, not in having an absolute requirement for a nutrilite, but in having a longer lag-phase without it.

The present paper deals with the isolation, after X-ray treatment of dried cells, of mutant strains of *Aerobacter aerogenes* which have growth-factor requirements additional to those of the parent strain (Beadle, 1945). The growth-factor requirements of most of the mutants were partially identified. Mutants were isolated which required one of the following substances, or substances metabolically related to them: aneurin, methionine, adenine, uracil, histidine, threonine, arginine, nicotinic acid, glycine, asparagine. The parent strain did not require any of these and grew well on a medium in which the only organic compound was glucose.

EXPERIMENTAL

Strain. The micro-organism used was a non-motile strain of *A. aerogenes* (AlOC) originally isolated by one of us (C. H.) from dried milk. It was purified by single cell isolation, and kept pure by occasional single colony isolations. It grew well on the defined medium (referred to as the 'minimal' medium) used by Lodge & Hinshelwood (1943).

Media. The 'minimal' medium consisted of: $(NH_4)_2SO_4$, 0·96 g.; KH_2PO_4, 3·46 g.; $MgSO_4 . 7H_2O$, 0·04 g.; glucose, 20·0 g., all dissolved in 1 l. distilled water, the pH being adjusted to 7·2 with sodium hydroxide.

The 'complete' medium consisted of: peptone, 10·0 g.; yeast extract (Yeastrel), 5·0 g.; glucose 10·0 g.; tap water, 1,000 ml.; pH 7–7·2. Both media were solidified, when necessary, with 1·5 % of agar. For special purposes, both media were modified as mentioned below.

Irradiation. Irradiation was carried out at 7·5 cm. from the tungsten target of an air-cooled Coolidge tube (Watson Mobilix X-ray unit with Aeromax 12 tube); peak KV 80, 5 mA; output on the specimen about 1,500 roentgen/min. The dose was varied by varying the time of exposure.

The drying of cells for irradiation. Cells grown (24 hr. at 37°) on agar slopes of complete medium were suspended in complete medium (diluted 1/1), centrifuged for 1 min. at low speed to remove clumps, and the supernatant liquid adjusted to give a Spekker absorptiometer reading equivalent to a viable count of 12 to 14×10^7 cells/ml. Of this suspension 0·01 ml. was pipetted on to a sterile disk of Cellophane 600 (permeable) 1 cm. in diameter; several such disks were placed in a sterile Petri dish and cooled for 1 hr. in the refrigerator. They were then transferred to a cooled vacuum desiccator over anhydrous calcium chloride and the pressure kept at 1 mm. Hg for 2 hr. An average of 33 % of the bacteria survived this treatment. When stored in a desiccator at atmospheric pressure and room temperature 50–70 % of the bacteria which had survived the drying on these disks were still alive after three weeks.

Determination of nutritional requirements by auxanography

A 24-hr. culture of a mutant in liquid complete medium was centrifuged, washed twice with sterile ¼ strength Ringer's solution, and a loopful of the third suspension spread on minimal medium agar plates and dried. A few minute crystals of each of the vitamins, amino-acids, purines and pyrimidines to be tested were put in marked positions on the inoculated agar surface and the plates incubated at 37°. As the parent culture would not grow on minimal medium alone, good confluent growth at any locus on the plate was taken to indicate that the mutant strain required as a nutrient that particular substance corresponding to the site of growth (Beijerinck, 1889; Pontecorvo, 1947, 1949). This was confirmed in liquid minimal medium to which the particular substance was added.

Irradiation of dried cells

Cellophane disks carrying a dried film of bacteria were placed in a small sterile Petri dish, the lid of which was made of Cellophane, and were irradiated with dosages of 135,000 or 180,000 r. administered in 90 and 120 min. respectively. (By a mistake in calculation the dosages were stated as 35,000 and 50,000 r. in the preliminary report of Devi, Pontecorvo & Higginbottom, 1947.) Several untreated disks were used as controls. The dried films of bacteria, from treated and control disks, were resuspended in ¼ strength sterile Ringer's solution, the suspension diluted to give 50–200 colonies/plate and plated. The technique of isolation was that of Lederberg & Tatum (1946). With this technique presumptive mutant colonies are identified as delayed colonies which come up after the addition of a layer of complete medium, or one containing one or more growth factors, to plates initially prepared with minimal medium.

Both the complete and the minimal media given above had to be modified to check the vigorous gas production which otherwise cracked the agar in such layer plates. Glucose was entirely omitted from the complete medium and in the minimal medium the ammonium sulphate was increased to 5 g./l. and the glucose decreased to 5 g./l.

The suspension of cells (treated or control) was added to the middle layer of dishes plated with three layers (about 5 ml. each) of modified minimal medium.

After incubation of the plates at 37° for 36–38 hr. the colonies then visible were counted and marked on the back of the dish with spots of Indian ink. These colonies, having developed in minimal medium, were presumably produced by non-mutated bacteria. A fourth layer, of modified complete medium agar, was then added, and the plates incubated for a further 24 hr. A further small number of colonies usually developed and these were all isolated and tested. Only a few of these delayed colonies were found to be permanent mutants persistently unable to grow on minimal medium unless supplemented.

Table 1. *The results of irradiating dried cells of* Aerobacter aerogenes *with X-rays*

	No. of colonies which grew			
Irradiation dosage (roentgens)	Before addition of complete medium (*a*)	After addition of complete medium (*b*)	Confirmed mutants among (*b*)	Code number of each mutant
135,000	100	6	1	3A3
	1220	27	5	4A3, 4, 10$^+$, 15$^+$, 31
	122	2	1	5A2
	1541	7	2	7A2$^+$, 4$^+$
180,000	272	8	1	5B8$^+$
	735	15	4	7B1$^+$, 2$^+$, 3, 5$^+$
	1468	17	6	9B1, 2$^+$, 4$^+$, 6$^+$, 16$^+$, 17
	198	10	0	
	1364	18	3	11B1, 2$^+$, 16
	1641	11	1	12B19
Total	8661	121	24	
Non-irradiated controls				
0	181	0	0	
0	555	10	1	5C1
0	872	6	0	
0	889	8	0	
0	1809	4	0	
0	2097	9	0	
0	1728	17	0	
Total	8131	54	1	

$^+$ Denotes adaptable mutants.

The results of a number of experiments (Table 1) show that of 8,185 colonies from non-irradiated dried bacteria only one was a mutant, while of 8,782 colonies from irradiated dried bacteria twenty-four were mutants. It is clear that irradiation increased the proportion of mutant colonies; but of the delayed colonies, which became visible only after the addition of complete medium, only a small proportion were mutants. Despite this, the stratification technique is certainly labour-saving; it involves the isolation of the delayed colonies only, among which the proportion of mutants in our experiments was about one in six. In preliminary experiments in which all colonies from irradiated cells plated on complete medium were isolated the proportion of mutants was usually well below one in a hundred.

An attempt to assess the survival rate after irradiation was made, but the results were too variable from experiment to experiment to allow a reliable estimate; in the ten series of irradiations tabulated in Table 1, the survival rate varied from 0·1 to 2·0/1,000. These rates were calculated by comparing, on complete medium, the viable counts of suspensions from non-irradiated disks of dried bacteria with the viable counts from an equal number of irradiated disks. Two main sources of variation were noted: (*a*) the initial number of viable bacteria on each disk, (*b*) the viable counts on the same suspension *after irradiation* according to the medium used for plating. No further attempt was made to standardize this part of the technique, and therefore no estimate could be made of mutation rates.

Table 2. *The classification of 25 mutants according to growth-factor requirements*

Growth-factor used by the mutant	No. of mutants	Mutant No.	
		Non-adaptable	Adaptable
Nicotinic acid	1	3A3	.
Histidine	1	.	9B2
Uracil	2	5C1	4A10
Methionine	1	4A4	.
Serine or Glycine	1	7B3	.
Threonine	1	9B1	.
Arginine	1	12B19	.
Adenine	1	4A31	.
Aneurin	3	11B16	4A15, 7A2
Asparagine	1	.	11B2
Thiosulphate	2	4A3	7A4
Yeast extract	9	5A2, 9B17	7B1, 7B2 7B5, 5B8, 9B4, 9B6, 9B16
Unknown (strain lost)	1	11B1	.

Types of mutants obtained

In all, 25 mutants were obtained, of which one was from non-irradiated bacteria and 24 from irradiated bacteria. As shown in Table 2, for 15 of the 25 mutants it was possible to identify one or more substances which allowed growth to occur when added as a supplement to the minimal medium. Of the remaining 10 strains, one (11B1) was lost and for the others attempts to find the additional substance or substances required for growth were unsuccessful. Failure may indicate either a multiple requirement or a requirement for substances other than those included in our tests.

The mutants obtained were of two main types; all required for immediate growth the addition to the minimal medium of a particular compound or a complex nutrient mixture such as yeast extract. Some, however, eventually grew even without this addition after a lag of 24 hr. or more; these are described here as 'adaptable'. Thirteen of the 25 mutants were of the adaptable type. Preliminary experiments on a number of these adaptable mutants revealed the complexity of the process of adaptation, which has been emphasized by all careful workers. Taken at their face value our results are compatible with a mechanism of reversible mutation from growth-factor dependence to independence and vice versa, coupled with differential growth rates such as to lead to an equilibrium between dependent and independent cells, the position of equilibrium varying with the medium. The work of Ryan (1946, 1948) has shown how delicately adjustable a mechanism like this may be. However, other possibilities cannot be ruled out, but since our experiments on this aspect of the work were not conclusive we refrain from reporting them here.

Table 3. *Alternative growth-factors tested with* 13 *mutants*

Growth-factor first identified	Strain	Other substances tested and found to be	
		(*a*) effective	(*b*) ineffective
Nicotinic acid	3A3	Nicotinamide	Anthranilic acid, indole, tryptophan, quinolinic acid, picolinic acid, pyridine, piperidine, ornithine, proline, α-amino-n-valeric acid, trigonelline, kynurenic acid, 3-hydroxy-anthranilic acid.
Histidine	9B2	.	4-Hydroxy-methyl glyoxaline alone or plus alanine or glycine, anserine, benziminazole.
Methionine	4A4	.	Choline, cystine, cysteine, thioformamide, cystine plus choline, taurine, thiolacetic acid, thiodiglycol, sulphanilic acid, phenylthiourea.
Serine or glycine	7B3	.	Alanine, β-alanine, pyruvic acid, propionic acid, acetate.
Threonine	9B1	.	α-Aminobutyric acid, sodium butyrate.
Arginine	12B19	Ornithine, citrulline	Glutamic acid, proline, guanidine and α-amino-n-valeric acid (singly and together), ammonium carbamate, urea, creatinine.
Adenine	4A31	Adenosine, adenylic acid, guanine, guanosine, guanylic acid	Cytidine, uracil, thymine, uric acid, allantoin, 2, 4, 5,-triamino-6-hydroxy-pyrimidine, benziminazole.
Aneurin	11B16	Vitamin thiazole	Thioformamide, vitamin pyrimidine.
Aneurin	4A15 7A2	Vitamin thiazole, vitamin pyrimidine, thioformamide	—
Thiosulphate	7A4	Metabisulphite, hyposulphite, methionine, cystine, cysteine, thioformamide, thiourea, thiolacetic acid	Persulphate, sulphite, sulphate.
Thiosulphate	4A3	As 7A4, sulphite	Persulphate, sulphate.
Uracil	5C1	Cytidine, cytidylic acid	Thymine, uric acid, allantoin.

The information on the growth responses of the mutants is summarized in Table 3. Of the 15 mutants for which a necessary growth-factor was identified 13 were further tested for their ability to grow with alternative substances. Unfortunately, not many of the substances which should have been tested were available at the time this work was done. However, from inspection of Table 3, the following remarks can be made.

Reduction of sulphate. The two strains 7A4 and 4A3 suggest two different blocks in the reduction of sulphate; 7A4 was unable to utilize sulphite but grew on more reduced compounds, while 4A3 was able to utilize it (cf. Fries, 1946, for *Ophiostoma*; Hockenhull, 1948, for *Aspergillus*).

Biosynthesis of nicotinic acid. For strain 3A3 anthranilic acid, 3-hydroxyanthranilic acid, indole, tryptophan and kynurenic acid were unable to replace nicotinic acid. The block in the biosynthesis of nicotinic acid in this mutant may occur at some late stage in the synthesis of nicotinic acid as with some of the mutants of *Neurospora* described by Yanofsky & Bonner (1950).

Biosynthesis of adenine. The mutant 4A31 could utilize guanine instead of adenine, a property which it shares with some mutants of *B. subtilis* (Burkholder & Giles, 1947) but not with the numerous mutants of *Ophiostoma* (Fries, 1946), *Neurospora* (Mitchell & Houlahan, 1946) or *Aspergillus nidulans* (Pontecorvo, unpubl.)

Biosynthesis of aneurin. The non-adaptable mutant 11B16 could utilize the thiazole moiety of aneurin, but not the pyrimidine moiety; it could not use thioformamide in place of the thiazole. The lag-phase of the two adaptable mutants 4A15 and 7A2 was shortened by the presence of either moiety of aneurin or by thioformamide.

Conclusions

The present work has shown that bacterial cells irradiated in the dry state can give rise to nutritional mutants. Taken together with the results of Burkholder & Giles (1947) on the irradiation of bacterial spores, this confirms that active growth and division at the time of irradiation are not essential conditions for mutation induced by X-irradiation. About half of the mutants obtained differed from the parent strain not by an absolute dependence on specific nutrient but in showing an extended lag in the absence of it. The mechanisms of this extended lag might be of the kind suggested by Hinshelwood (1946), of the kind usually referred to as mutation-plus-selection, or of other types, as suggested by the work of Sonneborn (1946) with *Paramecium* and of Ephrussi, Hottinguer & Tavlitzki (1949) with yeast.

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The Haemolysin of *Bacterium coli*

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SUMMARY: Conditions for haemolysis by *Bacterium coli* have been investigated. The haemolytic factor is specific to haemolytic strains only, has not at present been separated from living bacterial cells, is associated with the logarithmic phase of growth, and requires for its action the presence of calcium ions.

Except for an article by Dudgeon, Wordley & Bawtree (1921) suggesting a connexion between haemolytic and pathogenic effects, unconfirmed in ensuing years, the subject of haemolysis by *Bacterium coli* appears to have received little attention since the report by Kayser (1903) of a heat-resistant filterable haemolysin, particularly active upon dog red cells, which was produced during the later, more alkaline, phase of growth in an acid broth. A repetition of some of Kayser's experiments led me to the conclusion that his haemolysin was simply carbonate; and a re-examination of the haemolytic action of *Bact. coli* was undertaken, using chemically-defined media for growth.

METHODS

Ten strains of *Bact. coli* (seven haemolytic, three non-haemolytic for mammalian red cells, man, dog, horse, ox, sheep) two strains of *Aerobacter aerogenes* (non-haemolytic) and one Intermediate strain (non-haemolytic) were used in this work, all being from human sources (urinary infections or faeces) except the last, which was isolated from cow's milk. Cultivation at 37° was carried out in a defined minimal medium (Robinson, 1932) consisting of: sodium acetate ($NaC_2H_3O_2 . 3H_2O$), 2·5 g.; sodium citrate ($Na_3C_6H_5O_7 . 5H_2O$), 2·5 g.; NaCl, 5·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4 . 7H_2O$, 0·1 g.; N-NaOH 5·7 ml.; water to 1 l. After autoclaving, one vol. of sterile 10 % (w/v) ammonium sulphate solution was added to 100 vols. of the basal medium.

RESULTS

When defibrinated human blood or washed red cells were incorporated in the defined medium, or added to it after growth, no haemolysis occurred, although the same conditions would give rise to haemolysis in peptone water. Enrichment of the defined medium with 0·25 % Bacto-Peptone (Difco Laboratories Inc., Detroit) failed to produce the haemolytic phenomenon. The ingredients of the defined medium were, therefore, included separately (at usual concentration) in blood peptone water cultures. Haemolysis then occurred after overnight incubation with haemolytic strains of *Bact. coli* except in the medium to which sodium citrate had been added. Subsequent titration showed that, for complete inhibition of the haemolysis of one drop of defibrinated human blood in 8 ml. of 1 % Bacto-Peptone water, a minimal concentration of 0·2 % sodium citrate was required. The minimal completely inhibitory concentration of potassium oxalate was 0·03 %, and of sodium acetate and sodium lactate was 1–1·5 %. Partial inhibition occurred with concentrations of sodium citrate down to 0·1 %, potassium oxalate down to 0·008 % and sodium lactate and acetate down to 0·2 %. These observations suggested that calcium ions were needed for haemolysis. Even without inhibitors, the amount of calcium in the batch of 1 % Bacto-Peptone water used (estimated as 0·065 mg./100 ml.) was below the optimum, since addition of calcium chloride (0·1 %) enhanced the haemolysis.

A defined medium was then compounded in which *Bact. coli* was able to exert its haemolytic effect. A stock solution was made to contain (in %, w/v): $(NH_4)_2SO_4$, 10; KH_2PO_4, 0·5; NaCl, 5·0; $MgSO_4.7H_2O$, 0·05; citric acid $(5H_2O)$, 2·0; lactic acid (Analar), 4·0 v/v. For use, 100 ml. of this solution was diluted to *c.* 900 ml., then 20 ml. of 10 % $CaCl_2.6H_2O$ and 60 ml. of N-NaOH were added, the volume adjusted to 1 l., and the medium autoclaved at 120° for 30 min. When cool it was adjusted to pH 5·9 by adding *c.* 13 ml. of N-NaOH, distributed, and re-autoclaved at 115° for 15 min.

In this medium, with blood or washed red cells added before or after growth, five of the haemolytic strains of *Bact. coli* gave good haemolysis, which was abolished when 5 % $MgSO_4.7H_2O$ was used instead of 10 % $CaCl_2.6H_2O$ in making the medium. The two deficient strains (both non-motile) haemolysed more slowly than the others under the controlled conditions described later.

An attempt to separate the haemolysin from the living bacteria was unsuccessful. Heating suspensions at 60° destroyed the haemolytic power. Seitz-filtration, dialysis through cellophan, extraction of cultures with organic solvents and grinding or shaking active bacterial sediment with serum or strong peptone solution, all failed to yield demonstrable haemolysin, even in the presence of the reducing agent sodium dithionite which activates streptolysin O. Cultures of haemolytic strains, grown in peptone water or defined medium in collodion sacs suspended in saline containing blood, never gave haemolysis unless contamination of the outer fluid occurred.

Maximal haemolytic activity was found to occur during the logarithmic phase of growth. For studying the phenomenon, therefore, washed saline suspensions of bacterial cells were prepared (*c.* 10^9 cells/ml.) from young cultures which had undergone a preliminary series of rapid cultivations. Small screw-capped bottles containing *c.* 4 ml. peptone water, emptied and recharged with medium twice a day for three days after primary inoculation, gave a convenient seed for rinsing into a final volume of 10 to 100 ml., which was harvested after *c.* 16 hr. growth. Once it was realized that the slower rate of growth on defined media compared with peptone water was due mainly to oxygen lack, there was no difficulty, using Roux bottles, in activating suspensions in these media also. By this means it was shown that calcium was necessary only for the actual haemolytic process and not for the production of the haemolytic suspension.

The minimal haemolytic dose of such suspensions was *c.* 0·025 ml. when tested against 0·5 ml. of 5 % washed human red cells with 1·25 ml. saline and incubated at 37° in presence of 0·02 % $CaCl_2 . 6H_2O$, the time for complete haemolysis being *c.* 3 hr. With twice this dose, a freshly made suspension could give complete haemolysis in 1 hr., though this, the fastest time attained, required careful preparation of a strongly haemolytic strain. In all such haemolysis tests, the tubes were inverted every half hour to resuspend the red cells.

Nothing, it therefore appeared, beyond calcium, red cells and active bacterial cells, was needed to produce the phenomenon of haemolysis. In spite of the length of time of incubation which often preceded haemolysis, the tubes when haemolysing still showed the red colour of oxyhaemoglobin, though this would become faintly mauve if left at 37° overnight. When peptone was present in the tubes, reduction of haemoglobin was seen in a few hours, but was not related to the haemolysis which it succeeded or preceded according to the haemolytic activity of the bacterial suspension under test. Varying the pH by means of added buffers had negligible effects, provided the limits tolerated by the red cells themselves were not exceeded.

When the three essential components were varied in concentration, it was found that the ranges for producing haemolysis in 1·75 ml. total volume were: 0·005 to 2·0 mg. calcium, 10^6 to 10^8 bacterial cells, and any number (optimal 5×10^8 to 5×10^9) of red cells. Additional calcium was not necessary in tubes containing large numbers of red cells. These figures made it appear that one bacterial cell was capable of haemolysing 5,000 red cells under the given conditions, and suggested that multiplication might occur during the test, the red cells acting as medium. The effect of adding nutrients to the haemolysis test mixtures was, therefore, investigated: Bacto-Peptone (0·1 %), or sodium citrate (0·1 %) with and without $(NH_4)_2SO_4 + KH_2PO_4$, decreased the time of incubation preceding haemolysis; and in their presence the advantage of a high red cell concentration was abolished.

Thus it was indirectly shown that nutriment from the red cells was activating the haemolysis test described above. This was confirmed when saline washings of red cells, tungstate (Folin-Wu) blood filtrate or serum were also found to possess activating properties, retained after boiling and filtering. Substances such as sodium citrate, which are both carbon-containing and calcium-suppressing, exert inhibitory or activating effects according to their concentration in relation to that of calcium. The process of activation was also carried out by incubation of bacterial cells with activator before adding a suspension of red cells in calcified saline, and the haemolysis time was similarly decreased. But it was noticed that the process was most effective with the least active bacterial suspensions, i.e. usually those which had aged, and that if suspensions were very fresh the effect was decreased to a minimum. It therefore seems probable that the mechanism of activation is simply that of a last-minute subculture, throwing the cells back yet again into the logarithmic phase, in the same manner as the preparatory rapid subculturing which was found necessary to improve the haemolytic power of the suspensions.

Table 1. *Correlation of colony count and methylene blue reduction activity with haemolytic power of* Bact. coli *suspensions*

Haemolytic strain 7777/48 was used. Pre-incubation was carried out at 37° in two 6-in. × $\frac{3}{4}$-in. tubes in which 1 ml. of washed saline bacterial suspension (10^9 organisms/ml.) was diluted 1/10 in saline and in activator. Tubes of haemolysis test received 1 ml. suspension, 0·5 ml. 5 % washed red cells, 0·05 ml. 1 % $CaCl_2$ solution and 0·2 ml. saline.

Time of pre-incubation in saline (S) or activator (A) (hr.)	Relative numbers of colonies (48 hr., 37°) from dilution in agar plate	Time for complete lysis (hr.)	Approx. time for reduction of methylene blue in milk (hr.)
1. Fresh suspension			
0 (S)	600	1·0	5·5
0 (A)	500	1·0	5·5
1 (S)	700	1·25	5·5
1 (A)	400	0·75	5·5
2 (S)	400	1·25	5·5
2 (A)	300	0·75	5·5
4 (S)	800	1·25	5·5
4 (A)	400	1·25	5·5
24 (S)	10,000	3·00	7
24 (A)	10,000	3·00	7
2. Same, 24-hr. old			
0 (S)	1–2000	1·75	6
0 (A)	1–2000	1·5	6
1½ (S)	1–2000	2·0	6
1½ (A)	1–2000	1·0	6
3 (S)	1–2000	2·25	6
3 (A)	1–2000	1·25	6

An example of an experiment showing the instability and re-activation of haemolytic suspensions is given in Table 1, where, under the defined conditions given above, the haemolysis (complete) time is shown to be 1 hr. when the bacterial cells were washed and tested with a minimum of delay, and $\frac{3}{4}$ hr. after pre-incubation with activating solution (dilute citrate, Na_2HPO_4, NH_4Cl). Control pre-incubation in saline gave a slight lengthening of the time. Colony counts made at the same time-intervals as the successive haemolysis tests failed to show any significant change in numbers of viable bacteria due to pre-incubation in activating solution (which is not an adequate medium for growth of faecal *Bact. coli*) or saline, but they did show a considerable increase in numbers after 24-hr. pre-incubation, in saline as well as in activating solution, after which period the haemolysis time had lengthened to 3 hr. in both. The time taken by the suspensions to reduce methylene blue in milk was also investigated in this experiment, and found to vary rather with haemolysis time than with the increase in bacterial numbers, since at 24 hr., when the colony count had risen, the reduction time had slightly lengthened. The experiment was performed in duplicate with a non-haemolytic strain, whose results, omitted from the table, agreed with those of the haemolytic strain as regards : (1) absence of detectable increase in colony counts up to 4 hr. but increase at 24 hr.; (2) diminished reducing activity at 24 hr. when the colony count was higher.

The optimal haemolytic conditions of a relatively small dose of bacterial cells in the logarithmic phase, accompanied by activator, can be simply attained by centrifuging a young peptone-water culture of a haemolytic strain and adding the 'clear' supernatant to the tube of a haemolysis test (already containing calcium, saline and red cells) to give a 1/10 dilution. This finding may account for the report (Ponder & McLachlan, 1927) of a feeble haemolysin in the supernatant of centrifuged cultures of *Bact. coli.*

DISCUSSION

The common property of the activating materials examined appeared to be their nutritive effect. Ammonium salts and phosphates, which increased the activating effect of sodium citrate, were inactive without the latter substance. Concentrations of pH 8·3 borate buffer ($>0{\cdot}1$ M in tube of test) sufficient for permanent inhibition of growth were also inhibitory to haemolysis; while lower concentrations (*c.* 0·01 M), which scarcely delayed haemolysis, were sometimes noticed later to have permitted haemoglobin reduction. But activation could not be equated with growth in the ordinary sense of an increase in the number of bacterial cells; for haemolysis was delayed when more than the optimal dose even of a highly active suspension was used; while very thick suspensions, or those made from old cultures, reduced the haemoglobin without giving more than a trace of haemolysis. The difficulty of dealing with so unstable a system as a logarithmic-phase suspension has prevented more exact definition of haemolytic conditions. These suspensions appear to continue multiplying even in saline at room temperature, and it seems that the haemolytic power is connected with one particular time phase within the process, i.e. with one passing haemolytic generation. Refrigeration at 0° maintained the haemolytic power of suspensions at workable, though decreasing, levels for about three weeks. In addition to the methylene blue reduction test, other well-known metabolic actions of *Bact. coli* were investigated in parallel with haemolytic tests, but the generally high enzymatic activity of these haemolytic suspensions was shared by those of the non-haemolytic strains used as controls throughout this work.

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The Occurrence of Glutamic Dehydrogenase in *Neurospora* and its Apparent Absence in Certain Mutant Strains

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SUMMARY: A study (Fincham, 1950) of two mutant strains of *Neurospora crassa*, which probably owed their origin to independent occurrences of mutation at the same locus, showed them to be deficient in their ability to synthesize from ammonia the α-amino groups of a wide range of amino-acids. They grew well when any one of a number of different α-amino-acids was added to the medium, but would only grow slowly, and after a prolonged lag-phase, in the absence of amino-acid. The corresponding D-amino-acids and α-keto-acids were inactive in promoting growth. The mutants were subnormal in their capacity to assimilate ammonium nitrogen. In the present study the L-glutamic acid dehydrogenase activity of dialysed extracts of strains carrying the amination-deficiency mutation, and of a number of other wild-type and mutant strains, was investigated. All strains, with the exception of the amination-deficient strains, yielded an L-glutamic acid dehydrogenase specific for coenzyme II. Extracts of four amination-deficient strains had no detectable glutamic dehydrogenase activity, and did not inhibit the enzyme appreciably when added to wild-type extracts. Glucose-6-phosphate dehydrogenase was present in one extract tested for its presence.

Fincham (1950) described two mutant strains of *Neurospora crassa*, 32213 and 47305 of the series obtained by Beadle and co-workers, which appeared to have a metabolic block in the reaction normally responsible for the synthesis of α-amino groups from ammonia. The mutants, which differ from the wild-type in single Mendelian mutations probably at the same locus in each case, fail to grow on the minimal medium until after a prolonged lag period lasting 4–5 days at 25° and about 2 days at 35°. On medium supplemented with any one of the biologically occurring L-α-amino-acids, with the exceptions of glycine, serine, threonine, histidine, lysine and citrulline, growth begins without this lag, and a growth rate approximating to that of wild-type is achieved on media containing glutamate, aspartate, alanine, ornithine or arginine. The optimum concentration of these amino-acids is about 0·01 M, a very high requirement compared with that of most mutants which require amino-acids. A further property of these mutants is their very slow assimilation of ammonia as compared with wild-type under the same cultural conditions, and their tendency to accumulate ammonia when supplied with nitrate in the medium. It was suggested that the glutamic dehydrogenase system might prove to be absent or subnormal in strains 32213 and 47305. Evidence is presented in the present paper that this is indeed the case.

MATERIALS AND METHODS

Strains used. The wild-type strain P4-12 was obtained during breeding work by the author. It carries a spontaneous mutation which is cryptic at 25°, but which, at 35°, causes an enhanced requirement for carbohydrate (unpublished observations of S. Emerson and others).

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The wild-type strains E5256A and E5297a, derived by Dr Emerson, are known to be free of this mutation, the presence or absence of which has not been determined in the other strains used in this study.

Strain 481-34 carries a mutant gene causing an absence of the usual orange pigmentation in the spores and hyphae, and another mutation resulting in an inability to reduce nitrate to nitrite. This last mutation arose in *N. sitophila* and was transferred to *N. crassa* by repeated back-crossing from a species hybrid.

Strains 47305A and 32213,H1a were the mutant strains used in the author's original study, and the mutant strains 32213-8 and 47305-12 were derived from the crosses 32213,H1a × E5256A, and 47305A × E5297a respectively. For the sake of brevity, and to distinguish them from wild-type strains and other kinds of mutants, the amination-deficient strains 32213,H1a, 32213-8, 47305A and 47305-12 will be referred to as strains A_1, A_2, A_3 and A_4 respectively.

One culture of A_1 showed altered properties; amination-deficient strains derived from crosses made with this culture grew rather feebly on all media and were actually inhibited by ammonium salts. The spontaneous mutation responsible was separated from the amination-deficiency mutation by crossing some of the double mutants with a wild-type strain. Strains 88-1 and 88-4 were derived from such a cross: 88-4 is apparently a normal wild-type, but carries the modifying gene, as shown by a cross between it and a normal amination-deficient strain, while 88-1 does not carry the modifier (the other two spore pairs from ascus 88 gave, respectively, normal and 'inhibited' amination-deficient cultures).

All the other mutant strains used, the properties of which are summarized in the table, were obtained from the Pasadena laboratories by courtesy of Dr G. W. Beadle.

Culture methods. Mycelia for enzyme extractions were grown in Roux bottles each containing 100 ml. of the usual Fries No. 3 'minimal' medium (Beadle & Tatum, 1945) containing ammonium tartrate (0·5 %), glucose (1·5 %), biotin (5 μg./l.) and inorganic salts including trace elements, with appropriate supplements for the different mutants. A heavy inoculum of 1–2 ml. of a distinctly turbid suspension of conidia in sterile distilled water was used, and cultures were grown without agitation for 60–63 hr. at 25°, except where otherwise stated.

Stock cultures were kept on the same minimal medium solidified with agar and with the addition of yeast extract (0·5 %, w/v, 'Difco') and malt extract (2 %, v/v).

Determination of enzyme activity

In early experiments enzyme activity was followed by determination of ammonia, and, in one experiment, of α-ketoglutarate. Ammonia was estimated by the microdiffusion technique of Conway & Byrne (1933), followed by

Nesslerization, and α-ketoglutarate was estimated by the method described for α-keto acids by Sealock (1941). In most experiments, however, enzyme activity was shown by following the reduction and reoxidation of coenzyme II. This was done by measuring the ultra-violet absorption of digests at 340 mμ. with a 'Uvispek' spectrophotometer,

Preparation of coenzymes. Coenzyme preparations were obtained from horse liver using the methods developed for CoII by LePage & Mueller (1949). One liver yielded 570 mg. of a light brown powder from the first acetone precipitation, and this preparation was shown to contain, by a spectrophotometric assay following the method of LePage (1949), 22 % coenzyme calculated as CoI. Both CoI and CoII were presumed to be present in this preparation, and its behaviour in spectrophotometric experiments, to be described below, suggests that about 50 % of the coenzyme present was CoII. A 0·5 % (neutralized) solution of the preparation at this stage was used in a number of experiments. A further purification of the CoII by chromatography, as described by LePage & Mueller, yielded 31 mg. of a white powder assaying 44 % coenzyme, calculated as CoII. The amount of CoI present, though not determined with certainty, was probably low (see the section on coenzyme specificity). A specimen of CoI, assaying 55 % coenzyme calculated as CoI, was also obtained from the chromatographic column. The CoII preparation was made up as a 0·2 % neutralized solution for use, and was kept frozen for up to 10 days.

Enzyme extraction. Mycelium from a single flask was harvested on a large Buchner funnel, washed with *c.* 700 ml. distilled water and *c.* 80 ml. glass-distilled water, sucked fairly dry, pressed lightly between pads of towelling paper, and weighed. The mycelium was then ground with 2 to 4 vol. (usually 3 vol.) of 0·067 M phosphate (Na_2HPO_4—KH_2PO_4) buffer at pH 7·25 or (in a few experiments) pH 7·4, with the addition of powdered glass. Grinding was continued vigorously for 10 min. Both the mortar and the buffer were chilled to about 5° before use, and the mortar was stood in an ice bath during grinding.

In preliminary experiments in which enzyme activity was detected by ammonia estimations, the cream produced by grinding was centrifuged to get rid of cell-wall debris. The milky supernatant was free from large particles but contained many small particles of the order of size of bacteria (presumably mitochondria).

In experiments in which enzyme activity was detected spectrophotometrically, a particle-free extract was produced by filtering the cream produced by grinding through a pad of acid-washed kieselguhr on a small Buchner. At first a pad of 6–8 mm. thickness was used, but it was found difficult to suck such a pad sufficiently dry to avoid a considerable dilution of the enzyme during filtration (such a dilution did occur in the experiments the results of which are shown in Figs. 1, 2 and 3). Later a much thinner pad was found to be satisfactory, and a standard procedure was adopted as follows. Acid-washed kieselguhr (3·4 g.) was suspended in distilled water and poured on to a 2-in. diam. Buchner carrying a Whatman No. 50 filter paper. The pad was sucked fairly dry, pressed down to avoid cracks at the edges, washed through with glass-distilled water and dried by attaching to the water aspirator for 30 min.; this procedure gave a fairly dry pad about 3 mm. thick. The cream produced by grinding mycelium was filtered through this pad by suction. The last 2–3 ml. were retained rather tenaciously by the pad and were usually not collected. The filtrate was opalescent, but appeared free of particles under the 1/6 in. objective of the microscope. About 5 ml. of filtrate were dialysed against three changes of 250 ml. of the same buffer as used in the extraction, for 30–40 min. each change. During dialysis the external and the internal solutions were kept stirred, and the temperature of the whole was maintained at 0–2°. The dialysed extract was diluted 1/10 for use.

Both the particle-free extracts and extracts containing 'mitochondria' were found to contain a glutamic dehydrogenase (see next section). One attempt was made at a further purification of the enzyme preparation. A protein fraction was precipitated from a dialysed particle-free extract of strain E5297a (wild-type) by the addition, with stirring, of ethanol to a concentration of 40 % (v/v) at −5°. The precipitate was centrifuged down and redissolved in 0·067 M phosphate buffer at pH 7·25. This preparation showed glutamic dehydrogenase activity (tested for spectrophotometrically), though less than that of the original dialysed extract. A protein fraction precipitating between 40 and 80 % (v/v) ethanol had no detectable glutamic dehydrogenase activity.

RESULTS

Enzyme activity measured by ammonia estimation

Glutamic dehydrogenase activity was first detected in the extracts of wild-type (E5297a) mycelium obtained by grinding and centrifuging. When digests containing 1 ml. of E5297a extract buffered at pH 7·25, with NH_4Cl added to give an ammonia concentration of about 4 μM./ml., 0·15 ml. of 0·2 M sodium α-ketoglutarate, and 0·3 ml. of a 1 % neutralized solution of a crude liver nucleotide fraction (assaying 7 % coenzyme, calculated as CoI), were incubated for 30 min. at 30° in evacuated Thunberg tubes, 50–70 % of the ammonia originally present disappeared. In the absence of either the α-ketoglutarate or coenzyme there was a small increase in ammonia during the experiment. The reaction proceeded aerobically, but rather more slowly than in evacuated Thunberg tubes. Using ammonia disappearance in the presence of α-ketoglutarate as the test for glutamic dehydrogenase activity, the enzyme was detected in extracts of E5297a mycelium grown on minimal medium or on minimal medium supplemented with either 0·02 M DL-alanine or 0·01 M L-glutamate. The degree of activity shown, however, was not always consistent from experiment to experiment, and in a few cases no activity was found.

In one experiment, using a dialysed extract of E5297a, disappearance of α-ketoglutarate was demonstrated, and the production of glutamate was shown by the appearance of a strong glutamic acid spot in an ascending paper chromatogram of the deproteinized reaction mixture, phenol saturated with water being used as the running solvent.

Enzyme activity measured spectrophotometrically

(a) *Experiments using the CoI+CoII preparation*

In these experiments the cruder preparation mentioned under 'Materials and Methods' (assaying 22 % coenzyme, calculated as CoI) was used. Addition of crude coenzyme solution to dialysed, particle-free extracts (buffered at pH 7·25) of either E5297a or A_2 led to a rapid reduction of the coenzyme. The increase in absorption observed tended to about 50 % of the theoretical value for complete coenzyme reduction. The coenzyme was not reduced appreciably by boiled extracts.

In four experiments using extracts of E5297a mycelium, grown in one case on minimal medium, in one case on minimal with 0·01 M L-glutamate, and in two cases on minimal with 0·02 M DL-alanine, reduced coenzyme was almost completely reoxidized by the addition of α-ketoglutarate and ammonia, each to a final concentration of 0·005 M. When ammonium chloride and α-ketoglutarate were present in the digest before the addition of coenzyme, little or no reduction of coenzyme took place. α-Ketoglutarate alone had some effect, and the addition of α-ketoglutarate was more effective in decreasing the final degree of coenzyme reduction attained than was the addition of an equivalent amount of ammonium chloride. Addition of ammonium chloride to digests containing α-ketoglutarate, or of α-ketoglutarate to digests containing ammonium chloride, caused almost complete reoxidation of coenzyme. Results of a representative experiment of this type are shown in Fig. 1.

In three experiments using A_2 mycelium, grown in one case on minimal with 0·02 M DL-alanine, and in two cases on minimal with 0·01 M L-glutamate, no effect of added ammonia on the degree of coenzyme reduction was found. The results of one of these experiments are shown in Fig. 2. α-Ketoglutarate inhibited the reduction somewhat, but this inhibition was independent of ammonia concentration. The α-ketoglutarate appeared to have the effect of lowering the concentration of reduced coenzyme with which the system was in equilibrium, since not only did its presence cause the cessation of coenzyme reduction at a lower concentration, but its addition to digests after the coenzyme had become reduced caused a reoxidation to the same concentration of reduced coenzyme as was attained in digests to which α-ketoglutarate was added at the start (Figs. 2 and 3 show this phenomenon). This effect of α-ketoglutarate is unexplained, but its independence of ammonia indicates that it cannot be due to the presence of glutamic dehydrogenase.

One extract was prepared from mycelium of strain A_2 which had been allowed to grow on unsupplemented minimal medium for six days. The results of an experiment with this extract are shown in Fig. 3, and are essentially the same as those obtained using A_2 mycelium grown with an amino-acid in the medium.

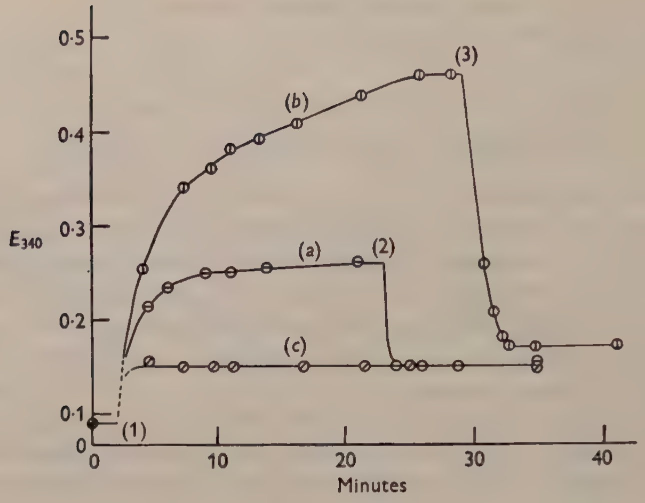

Fig. 1. Coenzyme reduction by extracts of E5297a (wild type) mycelium grown on minimal medium + 0·01 M-Na L-glutamate. 4·9 g. wet weight of mycelium ground with 12 ml. 0·067 M phosphate buffer at pH 7·25, filtered through kieselguhr, dialysed against the same buffer, and diluted with 5/7 vol. of buffer. Spectrophotometer cells (a), (b) and (c) each contain 1·5 ml. of diluted extract and receive, respectively, 0·1 ml. 0·1 M.-Na α-ketoglutarate + 0·1 ml. water, 0·1 ml. water + 0·1 ml. 0·1 M-NH_4Cl, and 0·1 ml. 0·1 M α-ketoglutarate + 0·1 ml. 0·1 M-NH_4Cl. 0·15 ml. of 0·5 % solution of the CoI + CoII preparation added to each cell at (1), at (2) 0·1 ml. 0·1 M-NH_4Cl added to (a), and at (3) 0·1 ml. 0·1 M-Na α-ketoglutarate added to (b). Absorption values are for a vol. of 1·85 ml.; corrections have been made for dilution and for α-ketoglutarate absorption. The absorption of unreduced coenzyme is approximately 0·04. Temperature 22–23°.

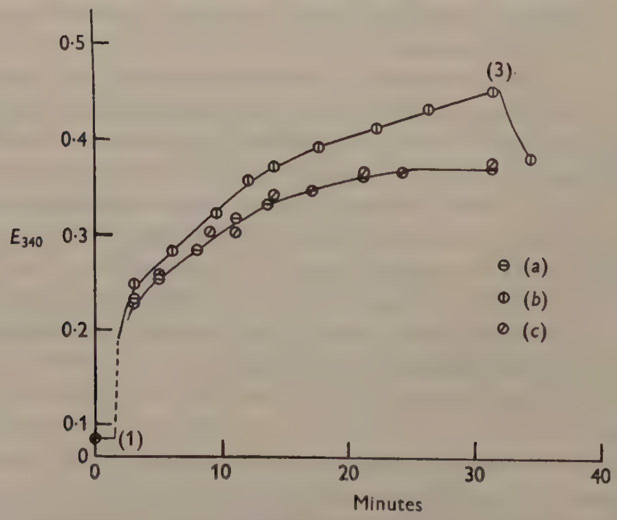

Fig. 2. As for Fig. 1 except that an extract of A_2 mycelium grown on minimal medium + 0·01 M-Na L-glutamate used 2·8 g. of mycelium ground with 8 ml. buffer, filtered, dialysed and diluted with 3/4 vol. buffer. Temperature 22–23°.

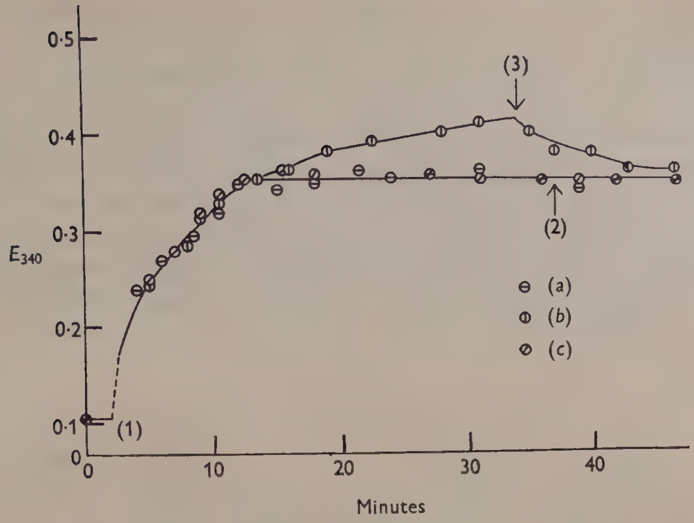

Fig. 3. As for Figs. 1 and 2, except that extract is of A_2 mycelium grown on minimal medium (unsupplemented) for 142 hr. 1·45 g. mycelium ground with 6 ml. buffer, filtered through a rather thick (*c.* 8 mm.) pad of kieselguhr (becoming considerably diluted), dialysed, and used without further dilution. Temperature 22°.

(b) *Experiments using the CoII preparation*

Reduction of coenzyme in the absence of glutamate. When the CoII preparation was used instead of the cruder CoI+CoII preparation, the extent to which the coenzyme was reduced by dialysed extracts alone varied from experiment to experiment. The rapidity of reduction of coenzyme in the absence of added glutamate appeared to be a function of the strain used and also of the cultural conditions. Extracts of strains 299, 66702 and E5041 (see table) gave very slow reduction in the absence of glutamate, while other strains all gave more or less higher rates. The amination-deficient strains A_2, A_3 and A_4 all gave rather high control rates of reduction in experiments run at pH 7·25, although an extract of A_4 reduced coenzyme rather slowly in an experiment at pH 7·4 (Fig. 4). An extract of E5297a mycelium grown for 48 hr. at 37° showed a very high rate of reduction of coenzyme even though it was subjected to prolonged dialysis (overnight). No reduction of coenzyme occurred when part of the same extract, after having been heated to 100° for a few seconds, was used. It is thought probable that the reduction in the absence of glutamate was due to an enzyme, present to different degrees in different extracts, which oxidizes some substrate present in the CoII preparation. No information on the nature of this substrate is available, except that it appears to form part of a CoII-linked system (see below).

Glutamic dehydrogenase activity in different strains. The routine test for glutamic dehydrogenase activity using the CoII preparation was as follows. Two 1 cm. spectrophotometer cells each received 1·5 ml. of buffered extract and, respectively, 0·3 ml. of 0·5 M monosodium glutamate, and 0·3 ml. of water. Then 0·1 ml. of the 0·2 % solution of the CoII preparation was added to each, and its reduction was followed spectrophotometrically. When the reaction appeared to be approaching equilibrium 0·05 ml. of 0·1 M α-ketoglutarate was added to each cell, followed, after some minutes, by 0·1 ml. of 0·1 M ammonium chloride. It should be noted that, owing to the fact that α-ketoglutarate has itself a significant absorption at 340 mμ., a correction had to be applied to all readings where α-ketoglutarate was present. The size of the correction was determined by measuring the increase in absorption following the addition of 0·01 M α-ketoglutarate to the experimental reaction mixtures with water substituted for coenzyme solution.

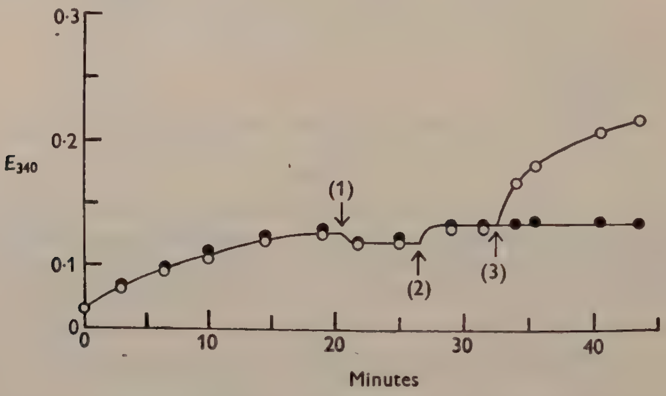

Fig. 4. Experiment on an extract of A_2 mycelium grown on minimal medium + 0·01 M-Na L-glutamate. 3·3 g. mycelium ground with 10 ml. of 0·067 M phosphate buffer at pH 7·6, filtered through 3 mm. of kieselguhr, dialysed, and diluted with 9 vols. of the same buffer. Spectrophotometer cells (a) (solid circles) and (b) (open circles) each receive 1·5 ml. diluted extract plus, respectively, 0·3 ml. 0·5 M-Na L-glutamate and 0·3 ml. water. 0·1 ml. of 0·2 % solution of CoII preparation added to each at time 0, 0·05 ml. 0·1 M-Na α-ketoglutarate at (1), and 0·1 ml. 0·1 M-NH_4Cl at (2). At (3) 0·05 ml. 0·12 M glucose 6-phosphate (Na salt) added to (b) only. Absorption values are calculated for a vol. of 1·9 ml., corrections being made for dilution and α-ketoglutarate absorption. Absorption of unreduced coenzyme is approximately 0·015. Temperature 18·5°.

Extracts of amination-deficient mutant strains reduced the coenzyme at the same rate, within experimental error, in the presence of added glutamate as in its absence. Some reoxidation of coenzyme followed the addition of α-ketoglutarate; this is doubtless the same phenomenon as was observed in the experiments using the cruder coenzyme preparation. The addition of ammonium chloride, however, caused no further reoxidation. Indeed, when allowance was made for the dilution effect of the addition of the ammonium chloride, it appeared in some cases to cause some reversal of the effect of α-ketoglutarate (Figs. 4 and 6). It seems clear that there is no evidence for the presence of glutamic dehydrogenase in these extracts. A representative experiment, using an extract of A_4 mycelium, is illustrated in Fig. 4.

Extracts of a considerable variety of other strains, including mutants and several wild-types of different origin, all gave clear evidence of glutamic dehydrogenase activity. The rate of reduction of the CoII preparation was always greater in the presence of glutamate than in its absence. The difference was very marked where the rate of reduction in the absence of glutamate was low, and less marked, but definite, where it was higher. Even where the effect of glutamate was rather small owing to the high rate of reduction in its absence, the effect of added ammonium chloride in causing an increased rate of reoxidation was always striking. The results of an experiment where the reduction in the absence of glutamate was intermediate in rate are shown in Fig. 5. The completeness of reoxidation following the addition of α-ketoglutarate and ammonium chloride depended on the rate of reduction in the absence of glutamate. Where this rate was low reoxidation was practically complete, while in the case of extracts where it was the highest, reoxidation proceeded only to the extent of about 60–70 % of the coenzyme originally reduced (see, for example, Fig. 6).

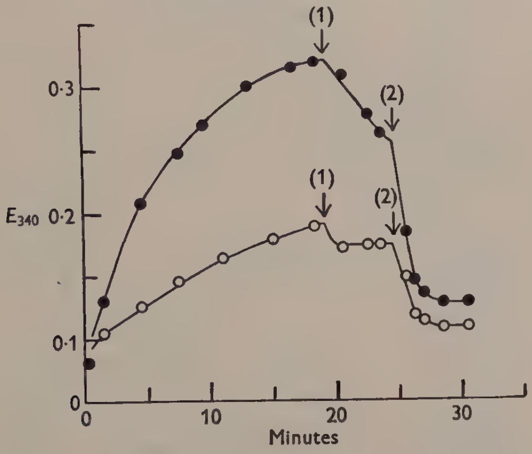

Fig. 5. As for Fig. 4 except that E5297a (wild-type) extract used. 3·5 g. mycelium ground with 10·5 ml. buffer at pH 7·6, filtered through 3 mm. kieselguhr, dialysed and diluted with 9 vols. of the same buffer. Temperature 18·5°.

In Table 1 the strains tested for enzyme activity using the CoII preparation are listed, together with the conditions under which they were grown. Owing to the lack of constancy in the rates of reduction in the absence of glutamate, only a rough comparison could be made between the degrees of glutamic dehydrogenase activity shown by the different strains. No differences in activity were apparent among the strains in which the enzyme could be demonstrated except in the case of strain 481-56, which yielded an extract with rather low, though still definite, activity. A point of particular interest here, as in the experiments using the cruder coenzyme preparation, is the lack of activity shown by amination-deficient strains grown on minimal medium for long periods. The negative result obtained with A_2 mycelium grown on minimal medium at 37° is regarded with some reserve; it is probable that the mycelium was growing very slowly when harvested, and the lack of opalescence of the extract indicated that the protein content was low. This extract, however, reduced coenzyme only very slowly, and even a very low glutamic dehydrogenase activity should have been detectable.

Extracts showing glutamic dehydrogenase activity lost their activity on being heated to 100° for a few seconds. Activity was, however, little if at all diminished by standing extracts for 2–3 hr. at room temperature, and it seems that some of the precautions observed to keep the extracts cold during preparation could probably have been omitted.

Table 1. *Mycelia tested for glutamic dehydrogenase using the CoII preparation*

Strain	Growth Requirements	Medium on which grown—supplements to minimal	Wet weight (g.)	Enzyme activity
E5297a	—	—	3·8	+
E5297a	—	—	3·5	+ (4)
E5297a	—	— (1)	?	+
32213-8 (A$_2$)	α-amino N	— (2)	0·9	—
32213-8 (A$_2$)	α-amino N	— (3)	1·1	— (4)
E5297a	—	0·01 M-Na L-glutamate	3·5	+
P4-12	—		3·7	+ (4)
32213-8 (A$_2$)	α-amino N		1·8	—
32213-8 (A$_2$)	α-amino N		2·4	—
47305-12 (A$_4$)	α-amino N		3·3	— (4)
47305A (A$_3$)	α-amino N	0·01 M-Na L-glutamate +1 μg./10 ml. pyridoxin	2·7	—
88-1	—		3·3	+
299 (*N. sitophila*)	Pyridoxin		3·3	+
P4-12	—	0·01 M-Na L-glutamate + 0·001 M L-arginine pyridoxin	3·0	+
47305-12 (A$_4$)	α-amino N		3·7	—
32213,H1a (A$_1$)	α-amino N		2·5	—
481-56	Nitrite or ammonium N		3·2	+
88-4	—		3·3	+
37803	Pyridoxin	Ditto but arginine 0·005 M	4·4	+
E5041 (6)	Proline, ornithine, citrulline or arginine	0·005 M L-arginine HCl	3·3	+
66702 (5)	Succinic or a related acid	0·005 M-Na succinate	1·2	+

Notes: (1) Grown at 37° for 48 hr. Dialysis overnight. (2) Grown at 25° for 139 hr. (3) Grown at 37° for 90 hr. (4) Tested at pH 7·6 (7·25 in all other cases). (5) Lewis (1948). (6) Srb, Fincham & Bonner (1950).

Coenzyme specificity. In two experiments using extracts of E5297a (wild-type) mycelium it was found that CoI was not reduced to a detectable extent by extract plus glutamate. In one experiment the CoI preparation obtained as a by-product of the CoII preparation was used, and in the other a specimen, of about 45 % purity, which had been prepared by Williamson's method. The same extracts reduced the CoII preparation rapidly in the presence of glutamate, and slowly in its absence. Thus the *Neurospora* glutamic dehydrogenase must be specific for CoII.

In some experiments it was shown that the reduction of coenzyme in the absence of glutamate was completely or almost completely reversed by the glutamic dehydrogenase system (Fig. 5), and in other experiments where the coenzyme added was practically all reduced (Fig. 6), up to 70 % was reoxidized by this system. From this it can be inferred that a system largely or entirely responsible for the reduction of coenzyme in the absence of glutamate also operates with CoII, and that the coenzyme present in the CoII preparation was at least 70 % CoII.

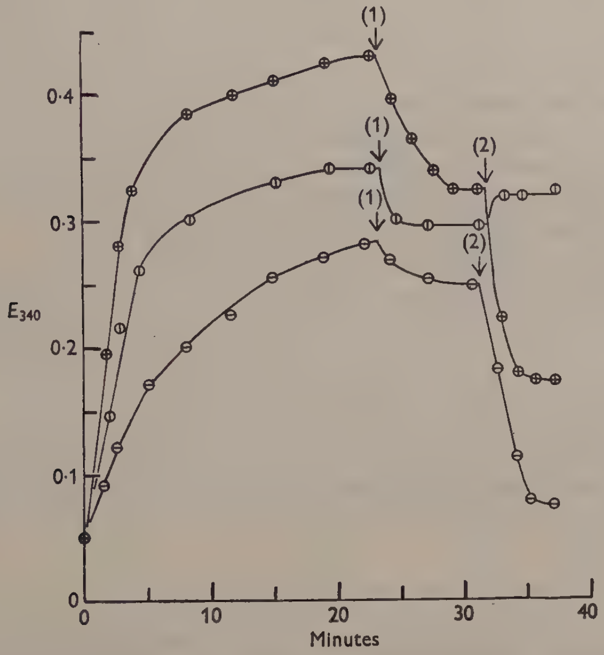

Fig. 6. Experiment on enzyme activity of a mixture of extracts of strains A_1 and 88-4, grown on the medium given in the table. 3·3 g. of 88-4 mycelium ground with 10 ml. 0·067 M phosphate buffer at pH 7·25, filtered through 4 mm. kieselguhr, and dialysed against the same buffer. 2·5 g. of A_1 ground with 7·5 ml. of the same buffer, filtered through 3 mm. kieselguhr, and dialysed similarly. Cell (a) (horizontal lines) contains 1·5 ml. of 88-4 extract diluted to 1/10 with buffer. Cell (b) (vertical lines) contains 1·5 ml. of A_1 extract diluted to 1/5 with buffer. Cell (c) (crosses) contains 1·5 ml. of a mixture of 1 part undiluted 88-4 extract: 2 parts undiluted A_1 extract: 7 parts buffer. Each cell receives 0·2 ml. 0·5 M-Na L-glutamate before the addition of 0·1 ml. of the 0·2 % solution of the CoII preparation at time 0. α-Ketoglutarate and NH_4Cl added at (1) and (2) as in Figs. 4 and 5. Corrections have been made for dilution and α-ketoglutarate absorption; readings plotted are for 1·8 ml. The increase in absorption observed in (c) (0·38) is close to the theoretical value for complete coenzyme reduction (0·37). Temperature 19·5°.

Activities of mixtures of extracts. It was thought possible that the lack of glutamic dehydrogenase activity shown by extracts of amination-deficient strains might be due to the presence of some inhibiting substance not easily got rid of by dialysis. To test this possibility, extracts of mutants having no activity were added to active extracts of wild-type strains in the expectation that any inhibitor present in the former extracts would cause a marked depression in the activity of the latter. The results obtained were not conclusive, in that the rate of coenzyme reduction in the absence of glutamate was increased by the addition of mutant extracts, and reoxidation would, in any case, be expected to be less rapid and complete where this 'background' rate of reduction was higher. However, as will be seen from Fig. 6, any inhibition caused by the mutant extracts must have been relatively slight. Four experiments of this type were carried out with essentially similar results in each case. One of these experiments involved the addition of two parts of the extract of strain A_2 grown at 37° (referred to above) to one part of an E5297a extract, the extracts being buffered at pH 7·4. In this experiment the 'background' rate of reduction was higher in the digest containing the mixed extracts than in that containing wild-type extract only, but reoxidation was practically complete in both cases and proceeded at the same rate, within experimental error. This experiment, however, was subject to the same reservations concerning the A_2 extract as mentioned earlier.

Glucose-6-phosphate dehydrogenase activity. In one extract the presence of this enzyme was demonstrated. The addition of 0·05 ml. of a 0·12 M solution of synthetic glucose-6-phosphate (sodium salt) to a digest containing extract of A_4 mycelium, buffered at pH 7·6, and CoII led to a rapid reduction of the coenzyme (Fig. 4). Further experiments showed that when 0·1 ml. of the 0·2 % CoII solution was added to 1·7 ml. of the same extract plus 0·05 ml. of the glucose-6-phosphate solution, about 85 % of the coenzyme was reduced, equilibrium being reached in 15 min. as compared with 25 % reduction in the same time in control cells with water instead of glucose-6-phosphate.

DISCUSSION

Though it is unfortunate that a more highly purified sample of CoII was not available, and that the complications due to the reduction of coenzyme in the absence of glutamate and the unexplained effect of α-ketoglutarate on this reduction were thus not avoided, the results of the spectrophotometric experiments seem clear. An active glutamic dehydrogenase, specific for CoII, was found in all strains of *Neurospora* tested except in four known to be carrying a mutation which results in a growth requirement for transferable α-amino nitrogen. The enzyme resembles, at least in its coenzyme specificity, the glutamic dehydrogenases of yeast and *Escherichia coli* (Adler, Günther & Everett, 1938; Adler *et al.*, 1938).

The results reported in the present paper cannot be taken as final proof that glutamic dehydrogenase is absent from the amination-deficient mutants. It remains possible that the enzyme may be present but is inhibited both *in vivo* and *in vitro* by some substance which inhibits the wild-type enzyme little, if at all, in mixtures of wild-type and amination-deficient extracts. Such a situation would imply some difference between the mutant and wild-type enzymes. As an alternative possibility, the inhibitor of glutamic dehydrogenase in the amination-deficient strains might be combined relatively indissociably with the enzyme, and not present in the free state to any significant extent in extracts. This condition might be difficult to distinguish from actual absence of enzyme. Yet another possibility, though perhaps rather a remote one, is that a glutamic dehydrogenase is present in the amination-deficient strains, but that, either because of its own peculiar properties, or because of some peculiarity in the nature or organization of other cell constituents, it cannot be obtained in active form by the extraction methods used in this work. Thus, though it seems probable that glutamic dehydrogenase is not produced by the amination-deficient strains, further work with a more highly purified system would be necessary to exclude alternative interpretations.

The work on the biochemical genetics of *Neurospora* has led to the conception (see, for example, Bonner, 1946) that single gene mutations have their effect through affecting the specificities of single enzymes. Although this hypothesis appears plausible, little good evidence in its favour has yet been forthcoming. Two cases in which an enzyme appeared to be affected by a gene mutation have turned out to be less simple than at first appeared. The first case concerned a pantothenic acid-requiring strain the intact mycelium of which could not be shown to possess the enzyme responsible for linking β-alanine and pantoyl lactone. Wagner (1949) has shown that the enzyme activity is readily obtained in *in vitro* preparations of the mutant. In this case, if the one gene-one enzyme relationship holds, there is no clue as to which enzyme is primarily affected. The second case, reported by Mitchell & Lein (1948), was one in which a strain requiring tryptophan for growth gave no tryptophan desmolase activity in extracts. According to a more recent report, however (Gordon & Mitchell, 1950), the mutant contains a tryptophan desmolase which is inhibited by certain substances normally present in the cell. The case reported in the present paper appears, at least on the surface, to be one in which the one gene-one enzyme hypothesis in its simplest form may hold. It is thought that further investigation of this case might cast some light on the gene-enzyme relationship by providing answers to such questions as, for instance, whether the mutants produce a protein similar to glutamic dehydrogenase, but lacking its specificity for coenzyme or glutamate.

The biochemical implications of this study seem rather more clear. The apparent absence of glutamic dehydrogenase in extracts of mutants having a general requirement for α-amino groups reinforces the suggestion, made in the author's previous report (1950), that, in *Neurospora*, the α-amino groups of all the amino-acids which support growth of the mutants are derived, at least to a very large extent, from ammonia via the glutamic dehydrogenase system. Such an origin for the α-amino groups of such a variety of amino-acids would require the existence of a wide range of transaminases such as has been recently shown to occur in bacteria (Feldman & Gunsalus, 1950) and animal tissues (Cammarata & Cohen, 1950).

It was previously shown by the author (1950) that the amination-deficient mutants are capable of assimilating inorganic nitrogen, even though their capacity to do so is impaired in comparison with wild-type. The failure to detect any glutamic dehydrogenase in mutant mycelium even when it had, after a prolonged incubation period, started to grow on minimal medium, suggests that this uptake of ammonia by the amination-deficient strains is due to some accessory amination mechanism which becomes especially developed under these circumstances.

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The Serology of *Bacillus polymyxa*

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SUMMARY: The spore, somatic and flagellar antigens of thirty-nine strains of *Bacillus polymyxa* have been investigated. The three antigens were serologically distinct and differed in thermal stability and in the appearance of the floccules after agglutination. The spore antigen was common to all the thirty-nine strains of *B. polymyxa* and appears to be species-specific. The somatic antigen was complex and absorption tests failed to show any clear-cut groups. On first isolation the organisms had flagellar antigens which were strain-specific, but after cultivation for some months on nutrient agar, an irreversible phase variation occurred which was associated with the appearance of group antigens. Five group-antigens have been recognized. One antigen was common to nearly all strains of *B. polymyxa* and also to the seven strains of *B. macerans* tested. Non-agglutinating antibodies were encountered in some flagellar antisera.

The only previous work on the serology of *Bacillus polymyxa* is by Porter, McCleskey & Levine (1937). Using heated vegetative cells as antigens they found that no one antiserum agglutinated all the strains although some antisera agglutinated 60% of the strains tested. No cross-reactions with strains of *B. macerans* were observed.

As part of a systematic study of the serology of the genus *Bacillus* an antigenic study of 39 strains of *B. polymyxa* has been made and the results are reported here.

EXPERIMENTAL METHODS

Source of strains

All the strains of the genus *Bacillus* used in this investigation were identified morphologically and biochemically by the methods described by Smith, Gordon & Clark (1946). Twenty-nine of the 39 strains of *B. polymyxa* were isolated by Francis & Rippon (1949). These strains were examined serologically immediately after isolation. Strains of the following species from the Wellcome Bacterial Collection were used in testing the *B. polymyxa* antisera for species specificity: *B. megatherium*, *B. cereus*, *B. cereus* var. *mycoides*, *B. subtilis*, *B. subtilis* var. *atterimus*, *B. subtilis* var. *niger*, *B. pumilus*, *B. coagulans*, *B. firmus*, *B. lentus*, *B. macerans*, *B. circulans*, *B. pantothenticus*, *B. alvei*, *B. laterosporous*, *B. brevis*, *B. pasteurii*, *B. sphaericus*, *B. sphaericus* var. *fusiformis*.

Preparation of antigens for immunization of rabbits

Somatic (O) antigens were prepared from selected smooth colonies. A subculture from a smooth colony in nutrient broth was incubated overnight at 28° and the growth resuspended in saline after centrifuging. The suspension was steamed for half an hour to destroy the H antigen and the opacity was adjusted to 8×10^8 organisms/ml. Cultures in broth were used in preference to agar cultures in order to avoid spore formation, which may occur during overnight incubation on agar, but has not been observed in broth even after several days' incubation.

Flagellar (*H*) *antigens* were prepared from motile 18-hr. broth cultures preserved with 0·25 % formalin (*c.* 0·1 % formaldehyde) and adjusted to the same opacity as the O antigen. The motility was increased as necessary by inoculating a semi-solid agar plate at the circumference and, after overnight incubation, subculturing into nutrient broth from the spreading edge of growth.

Spore antigens were prepared from five-day nutrient agar slope cultures of *B. polymyxa* which were examined microscopically to ensure that sporulation was complete. The growth was washed off the agar slopes with physiological saline and after steaming for 1 hr. was adjusted to 3×10^8 spores/ml. As *B. polymyxa* spores readily on nutrient agar, a suspension completely free from bacillary forms was easily obtained.

Preparation of antisera

Rabbits were injected intravenously at three-day intervals. Four doses of 1, 2, 3 and 3 ml. respectively were given to produce H antisera and a further three or four injections of 3 ml. each for the production of O and spore antisera. The antisera were preserved with 50 % (v/v) of glycerol and 0·01 % thiomersalate.

Agglutinable suspensions

The same procedure was followed as in the production of the antigens, except in the case of the H suspensions, which were prepared from broth cultures incubated at 37° instead of at 28°. Growth, though not so abundant at 37°, was less flocculent and gave stable suspensions for agglutination tests. Dense H suspensions for absorption tests were prepared from the growth on a semi-solid agar plate and preserved with 0·25 % formalin. H and O suspensions of other species of the genus *Bacillus* were also prepared from semi-solid agar plate cultures, as growth in broth often resulted in unstable suspensions. Spore suspensions of all species other than *B. polymyxa* were prepared from ten-day cultures on the asparagine medium described by Howie & Cruickshank (1940). A medium consisting of equal parts of asparagine agar and nutrient agar was sometimes preferable, as in some cases growth on asparagine medium was asporogenous.

Agglutination tests

The agglutination tests were performed in 8×1 cm. agglutination tubes containing 0·5 ml. of serum in a series of twofold dilutions, mixed with an equal volume of bacterial suspension. The tests were incubated at 54°. The H agglutination tests were read after 2 hr. and the O and Spore tests after overnight incubation. Incubation at 54° was preferable to 37°, as at the latter temperature suspensions containing much mucinous material were inagglutinable. This was particularly noticeable when non-motile O suspensions were not heated before use.

Absorption tests

Suspensions for absorption tests were of an opacity of *c.* 2×10^{10} organisms/ml. Equal volumes of antiserum and suspensions were mixed and incubated for 2 hr. at 54° and the absorbing suspension then removed by centrifugation. When the agglutinins to be absorbed were present in large amounts, the antiserum was diluted 1/10 or 1/100 before the addition of the absorbing suspension.

RESULTS

The H antisera had agglutination titres of 1/10,000, the O and Spore antisera 1/1,000 or more. The three antigens were distinct and no cross-reactions could be demonstrated between them and their respective antibodies. Antisera prepared from formolized H suspensions contained a little somatic agglutinin for the homologous strain. This was to be expected, and, as shown in Table 1, the O agglutinin can be removed by absorption, leaving the H titre unaltered. Absorption tests, on the other hand, showed that agglutinins were only removed by the homologous antigen. These results were confirmed with a number of strains of *B. polymyxa* and are essentially the same as those obtained by other workers using different species of the genus (Howie & Cruickshank, 1940; Lamanna, 1940*a*). Absence of bacillary agglutinins in the spore antisera is probably due to the ease with which completely-sporing suspensions free from bacillary forms can be prepared with this species. Rabbits injected with a spore suspension over a period of four months still failed to show any O agglutinins, although the spore titre was increased tenfold.

Table 1. *Reciprocal agglutinin absorption tests with* B. polymyxa *spore, O and H antisera and suspensions*

Anti-serum	Absorbing suspension	Agglutination titres with the following suspensions of the homologous strain		
		Spore	O	H
Spore	—	*1,600	0	0
Spore	Spore	0	0	0
Spore	O	1,600	0	0
Spore	H	1,600	0	0
O	—	0	800	0
O	Spore	0	800	0
O	O	0	0	0
O	H	0	800	0
H	—	0	100	2,560
H	Spore	0	100	2,560
H	O	0	0	2,560
H	H	0	0	0

* The titre is expressed as the reciprocal of the highest antiserum dilution to show visible agglutination.

0=No agglutination at an antiserum dilution of 1/10.

The appearance of the agglutination was characteristic for each of the three antigens. Floccular agglutination was characteristic of the H antigen and similar in appearance to that obtained with *Salmonella* H antisera and suspensions. The O and Spore agglutination was more granular and similar to the *S. typhi* Vi and O agglutinations, respectively. The H antigen also differed from the O and Spore antigens in being heat labile, being destroyed in 10 min. at 100° and inactivated by ethanol and chloroform. Saline suspensions of the O and Spore antigens still retained their antigenicity in rabbits after being autoclaved for 20 min. at 15 lb./sq. in. Similar results were obtained by Howie & Cruickshank (1940) using the spore antigen of *B. mesentericus*, but they found that the antigenicity *in vivo* of the vegetative bacilli was completely destroyed by autoclaving.

Spore antisera

Spore suspensions of all 39 strains of *B. polymyxa* were agglutinated by the seven S antisera prepared and were capable of complete absorption of all the homologous agglutinins. No cross-reactions have so far been observed with spore suspensions of other species of the genus. Table 2 lists the numbers of strains and species used.

Table 2. B. polymyxa *spore antiserum tested for specificity against spore suspensions of other species*

Test suspension species	No. of strains	Spore antiserum to *B. polymyxa* CN 2002 titre
B. polymyxa	(1, CN 2002)	1/800
B. polymyxa	39	1/400–1/800
B. megatherium	6	<1/10
B. cereus	13	<1/10
B. subtilis	8	<1/10
B. licheniformis	1	<1/10
B. pumilus	10	<1/10
B. coagulans	3	<1/10
B. firmus	3	<1/10
B. lentus	1	<1/10
B. macerans	8	<1/10
B. circulans	6	<1/10
B. alvei	3	<1/10
B. laterosporous	1	<1/10
B. brevis	8	<1/10
B. sphaericus	7	<1/10
B. pasteurii	3	<1/10

O antisera

O sera from 12 separate strains were prepared and the work of Porter *et al.* (1937) was in part confirmed. No one antiserum agglutinated all the strains tested, but a number of strains were agglutinated to 10 % of the homologous titre and others to 100 %. On absorption no clear-cut groups could be demonstrated, but in most cases there was a strain-specific agglutinin present.

Results of a typical agglutinin-absorption test are shown in Table 3. Brown, white and mucoid colonies described by Francis & Rippon (1949) gave similar agglutination results. Suspensions from rough colonies, however, were inagglutinable with antisera prepared from smooth colonies. An antiserum prepared from one strain of *B. polymyxa* in the rough phase agglutinated the rough variants of some strains of *B. polymyxa* but not those of other strains. Cross-reactions occurred between O antisera and O suspensions of a few strains of other species, including *B. lentus*, *B. subtilis* and *B. macerans*.

Table 3. *Representative agglutinin absorption tests with* B. polymyxa *O antiserum prepared from strain CN* 2002

Test suspension (CN no.)	Un-absorbed	Titre after absorption with O suspensions of strains						
		CN 2002	CN 1419	CN 1951	CN 2135	CN 2185	CN 1756	CN 2492
2002	*800	0	100	300	200	300	200	800
1419	800	0	0	0	200	200	50	800
1951	600	0	0	0	300	0	300	600
2135	800	0	0	200	0	300	0	800
2185	100	0	0	0	100	0	0	100
1756	800	0	0	0	400	0	0	800
2492	0	0	0	0	0	0	0	0

* The titre is expressed as the reciprocal of the highest serum dilution giving visible agglutination.

0 = No agglutination at a dilution of 1/10 of antiserum.

H antisera

H antisera were prepared from six freshly isolated strains. All these antisera agglutinated the homologous strain and no other. After maintaining these strains on nutrient agar for several months and then preparing fresh antisera for them, it was found that these new antisera agglutinated not only the homologous strain, but a number of other strains as well. The strains were kept as desiccates, some being dried immediately after isolation. These latter strains were still strain specific on subsequent recovery from the dried state. The recovered strains were plated and all the colonies appeared to be in the specific phase, but after a number of subcultivations on nutrient agar variation occurred, with the appearance of group antigens. Over a period of one year 35 of 39 strains showed this variation, which was not accompanied by any change in colonial appearance. This phase variation appeared to be irreversible. Attempts by the usual methods to obtain the specific variant from cultures in the group phase were unsuccessful. Table 4 illustrates this variation with five strains of *B. polymyxa*. The freshly isolated strains reacted only with their homologous serum, but these same strains after subcultivation on nutrient agar showed cross-reactions with other group antisera. It can be seen from Table 4 that in most instances the strain in the group phase still contained some specific antigen. However, with the strains CN 1419 and CN 1966 the variation was complete, the subcultivated strain containing only the group antigens. In strain CN 1966 there was an intermediate stage before

Table 4. *Phase variation in* B. polymyxa *H antigen*

Test suspension (CN no.)	Phase	Agglutination with antisera from strains									
		CN 2230		CN 2231		CN 1419		CN 2185		CN 1966	
		I	II	I	II	I	II	I	II	I	II
2230	I	+++	+++	—	—	—	—	—	—	—	—
2230	II	+++	+++	—	+++	—	++	—	+	—	++
2231	I	—	—	+++	+++	—	—	—	—	—	—
2231	II	—	++	+++	+++	—	+++	—	++	—	++
1419	I	—	—	—	—	+++	—	—	—	—	—
1419	II	—	++	—	++	—	+++	—	+	—	++
2185	I	—	—	—	—	—	—	+++	+++	—	—
2185	II	—	+	—	+++	—	+	+++	+++	—	±
1966	I	—	—	—	—	—	—	—	—	+++	—
1966	IIa	—	++	—	++	—	+	—	—	+++	+++
1966	IIb	—	++	—	++	—	+	—	—	—	+++

I, original culture on isolation (specific phase); II, culture after cultivation on artificial media (group phase); +++, agglutination at a dilution of 1/1280–1/2560 of antiserum; ++, agglutination at a dilution of 1/320–1/1280 of antiserum; +, agglutination at a dilution of 1/40–1/320 of antiserum; ±, agglutination at a dilution of 1/10–1/40 of antiserum; —, no agglutination at a dilution of 1/10 of antiserum.

the appearance of colonies containing only group antigens, when all colonies contained both specific and group antigens. This is represented by strain CN 1966 IIa in Table 4. Suspensions of organisms in the specific phase were incapable of removing group antigens by absorption. Suspensions of organisms in the group phase varied in their ability to remove agglutinins from antisera prepared against the specific phase depending on the extent to which variation had occurred. At least five group antigens have been recognized by absorption tests on antisera prepared from 22 strains in the group phase. One group antigen appears to be common to nearly all *B. polymyxa* strains tested, others are shared by two or more strains. A number of group antigens may be present in one strain. The results obtained by absorption tests are illustrated in Table 5, where it is shown that the antiserum prepared from *B. polymyxa* strain CN 2231 in the group phase contains some specific antibody and three group antigens, labelled for convenience A, B and C. Absorption tests showed that, of eight strains of *B. polymyxa*, one strain contained group antigen A, the group antigen common to most strains of *B. polymyxa*. Four strains contained group antigens A and B, and three strains contained antigens A and C. Two strains of *B. macerans* contained antigen A. These agglutination reactions were due to the flagellar agglutinating system, since the appearance of the floccules was characteristic and the agglutinability and absorbing power of the suspensions were lost when they were heated in a boiling water-bath for ten minutes.

Table 5. *Absorption tests on* B. polymyxa *CN* 2231 *H group antiserum*

Suspensions of strains (CN no.)	Group antigens present in suspension	Unabsorbed	Absorbed with CN 2231 in group phase	Absorbed with a mixture of strains containing group antigens*			
				A	A, B	A, C	A, B, C
2231	None (specific phase)	+†	—	+†	+†	+†	+†
2231	A, B, C	+++	—	++	++	++	++
1013‡	A	+++	—	—	—	—	—
1014		+++	—	—	—	—	—
2230		+++	—	—	—	—	—
1756	A, B	+++	—	++	—	++	—
1419		+++	—	++	—	++	—
2002		+++	—	++	—	++	—
2228		+++	—	++	—	++	—
2185	A, C	+++	—	++	++	—	—
2180		+++	—	++	++	—	—
2135		+++	—	++	++	—	—

* Mixture of strains used for absorption were the same strains as those shown in the left-hand column under their respective antigenic groups.

† Traces of specific agglutinin in the group phase serum of CN 2231.

‡ CN 1013, 1014 were strains of *B. macerans*, all other strains were *B. polymyxa*.

Cross-reactions between the *B. polymyxa* phase II antisera and H suspensions of other species of the genus *Bacillus* other than *B. macerans* have been observed, but only with a few strains of different species. These strains also reacted with sera prepared from strains of species other than *B. polymyxa*.

As the non-reacting strains were only agglutinated by their homologous sera, it seemed likely that these cross-reacting strains had already undergone the same kind of variation as occurs in *B. polymyxa*.

Non-agglutinating antibodies

During the preparation of group antisera from a number of different subcultures of the same strain, a phenomenon was observed for which no satisfactory explanation is offered. This effect is illustrated in Table 6. Antiserum A from CN 1419 in the group phase behaved normally and it was apparent that the seven other strains tested were of the same antigenic type. Absorption of the antiserum with any one strain removed the agglutinins to all the others. However, with antiserum B prepared from a different subculture of CN 1419 in the group phase, agglutination was obtained only with the homologous strain and low titre agglutination with one other, strain CN 1756. At first sight it appeared that the strain had reverted to the specific phase, but although the other strains were not agglutinated by the serum, they were capable of absorbing all the agglutinins. The strains which failed to agglutinate were also inagglutinable in the presence of 30 % bovine albumin or 10 % rabbit-serum. Blocking antibody of the type described by Griffitts (1947) for *Brucella* was, therefore, not present. Results of tests for incomplete antibody using hen anti-rabbit precipitating serum (Coombs, Mourant & Race, 1945; Morgan & Schütze, 1946) were equivocal. In some instances agglutination was obtained with anti-rabbit precipitating serum, but the method was not practical, due to the necessity of washing these suspensions free from rabbit serum while still retaining the flagella. A blocking test was carried out to see whether the normal agglutination between CN 1419 A antiserum and H suspensions of strains CN 2228 and CN 2184 could be prevented by previously incubating the suspensions with CN 1419 B antiserum. After 2 hr. incubation

Table 6. *Non-agglutinating antibodies in serum prepared from CN* 1419 *and* 2002 *in the group phase. Absorption tests with strains of the same serological group*

		Test suspension							
Anti-serum (CN no.)	Absorbing strains	1 CN 1419	2 CN 1756	3 CN 2228	4 CN 2184	5 CN 2222	6 CN 2369	7 CN 2370	8 CN 2002
1419 A	none	+++	+++	+++	+++	+++	+++	+++	+++
1419 A	*1–8	—	—	—	—	—	—	—	—
1419 B	none	+++	±	—	—	—	—	—	—
1419 B	1–8	—	—	—	—	—	—	—	—
2002	none	—	—	—	—	—	—	—	+++
2002	1–8	—	—	—	—	—	—	—	—

* 1–8 implies eight experiments in which the serum was absorbed by each strain separately. +++, agglutination at a dilution of 1/1280–1/2560 of antiserum; ++, agglutination at a dilution of 1/320–1/1280 of antiserum; +, agglutination at a dilution of 1/40–1/320 of antiserum; ±, agglutination at a dilution of 1/10–1/40 of antiserum; —, no agglutination at a dilution of 1/10 of antiserum.

with CN1419 B antiserum the suspensions were centrifuged, the deposit resuspended in saline and added to a dilution of CN1419 A antiserum. The suspensions were agglutinated to titre as before, with no trace of blocking effect.

DISCUSSION

By careful attention to cultural conditions, it is possible to prepare from strains of *B. polymyxa* suspensions which are suitable for agglutination tests and for differentiation of the spore, somatic and flagellar antigens. Under these conditions the Spore, O and H antigens have been shown to be distinct, thus confirming the observations of Mellon & Anderson (1919), Howie & Cruickshank (1940), and Lamanna (1940*a*), on other species of the genus. There has been little systematic serological investigation of the genus *Bacillus*. Lamanna (1940*a*), however, studied the spore antigens of several strains of *B. subtilis*, '*B. vulgatus*', '*B. mesentericus*' and '*B. agri*' and found that they could be divided into three well-defined serological groups. From the details in Lamanna's paper of their biochemical reactions, it seems likely that these organisms could be reclassified as : *B. licheniformis*, *B. subtilis* and *B. pumilus* (see Smith, Gordon & Clark, 1946; Knight & Proom, 1950). Each species has then a distinctive spore antigen. Lamanna (1940*a*) reported that the spore antigen appeared to be less complex than the bacillary antigens. Although Lamanna's results (1940*b*) with the spore antigens of the 'large-celled species' were less clear-cut, he suggested that a serological analysis would be of use in classification. The present work on *B. polymyxa* has shown that this species has a complex antigenic structure. The flagellar and somatic antigens are too numerous and complex to be of value in classification, but the spore antigen appears to be species-specific and of definite taxonomic value.

Heated spores were used as antigens in preference to living spores in order to avoid the formation of high-titre H agglutinins which were present in living-spore antisera after one injection. No O agglutinins could be demonstrated. The formation of H agglutinins may be due to the presence in the spore suspension of a few bacilli containing the highly antigenic H antigen. Krauskopf & McCoy (1937) working with strains of *B. subtilis* var. *niger* and Doak & Lamanna (1948) with *B. cereus* showed that the H antigen is present in the spore, but H agglutinins were only produced after the spores had been treated with potassium hydroxide. Whether or not the H agglutinins in the *B. polymyxa* living-spore antiserum are derived from the spore has not been ascertained. It seems likely that a study of the spore antigens covering the whole of the genus could be of considerable value; such work is now in progress.

The flagellar and somatic antigens of *B. polymyxa* appear to be too numerous to be of practical use in classification. The flagellar antigens can exist in both the specific and group phases. The somatic antigens are complex and several may exist together in one strain. Both O and H antigens may be shared by strains of *B. macerans*. It would be possible to characterize a number of different antigenic types in the O and H antigens, but such work would be of little practical value. Any grouping of strains by means of the O antigens has shown no relationship to the grouping based on bacteriophage sensitivity

(Francis & Rippon, 1949), or to the type of polymyxin produced (Brownlee & Jones, 1948).

Several points of interest have arisen from the results with flagellar antigens. With organisms freshly isolated from the soil, the flagellar antigen is invariably in the specific phase, but after the organism has been maintained on nutrient agar, an irreversible phase variation occurs, the speed of change depending on the strain. Some strains have undergone variation after a few subcultures while others remained in the specific phase after eight months' subcultivation at weekly intervals. There also appears to be another reaction occurring in the flagellar antigen which is responsible for the production of a type of 'non-agglutinating antibody'. This antibody combines with and agglutinates the homologous strain and can combine with, but not agglutinate, other strains of the same group phase. A serum may agglutinate some strains but not others, or may only agglutinate the homologous strain. No explanation is offered for this effect. It is not an 'incomplete antibody' in the true meaning of the term, since agglutination does not occur in the presence of 30% albumin and agglutination always occurs with the homologous strain.

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The Fermentation of Sugar-Beet Pulp Silage

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SUMMARY: An investigation was made of the processes occurring during the ensiling of sugar-beet pulp. Pulp was enclosed in rubber bags buried in a silo to obtain the same conditions of pressure and temperature as prevail in the silo itself. An outlet from the bags was provided for the measurement of evolved gases and seepage. Pulp alone was compared with pulp + 4 % molasses. The process was followed by withdrawing bags at short intervals, and making bacteriological and chemical examinations of the contents.

The addition of molasses resulted in a pure lactic acid fermentation of the sucrose with very small losses in dry matter. Few butyric acid bacteria were found. Where molasses was not added a vigorous butyric acid fermentation of hexosans and pentosans occurred, resulting in 12–15 % loss of dry matter due to seepage and formation of CO_2, the number of butyric acid bacteria rising to 10^9–10^{10}/g. silage.

The initial flora of lactic bacteria consisted of *Betacoccus arabinosaceus* Orla Jensen, which was soon displaced by *Streptobacterium plantarum* and *S. casei* Orla Jensen. After 2–3 months only *Betabacterium breve* Orla Jensen was found.

Sugar-beet pulp is a valuable by-product from the manufacture of beet sugar. It is poor in nitrogen (10 % total protein in the dry matter), but is an easily digestible and rather energy-rich feed when used in addition to protein-rich feeding stuffs or to the grass ration in summer; 1 kg. dry matter has a feeding value of about one feeding-unit. In Denmark, where about a million metric tons of sugar-beet pulp are produced yearly, it has a value as feed of about 25 million Danish kroner. It is thus worth while studying the conservation of these large amounts of fodder and to find ways of decreasing losses during storage. The most rational method of conservation is drying because losses due to fermentation and seepage are then avoided, but in most countries this method is too expensive, costs amounting to 20–30 % of the value of the pulp. Consequently in most countries pulp is conserved by ensiling.

Many experiments have been made to find the best ways to ensile beet pulp with the smallest possible losses. Most of these experiments have been conducted on a practical scale. Experiments by Steensberg (1945) showed that the addition of 2 % (v/v) of AIV-acid (equal parts of conc. HCl and conc. H_2SO_4) resulted in smaller losses and was slightly better than 1 % of molasses, which gave about the same result as 2 % formic acid (Amasil method). It was also found that ensiling a mixture of pulp and beet tops resulted in greater losses than their separate ensiling. Similar results were obtained in experiments at Swedish sugar factories where it was also found that hard pressing of the pulp, (raising the dry weight to 14–16 %) gave better results than the ordinary pressing to *c.* 10 % dry weight.

Jarl (1948) found that the digestibility of ensiled and dry pulp is about the same, although cellulose represents a greater part of the dry matter in the silage, and the feeding value of the dry matter of silage was greater than that of the dried pulp. Jarl (1948) also stated that the addition of 1, 2 or 3 % molasses was as good as, or even better than, the use of AIV-acid. The costs are about the same, but molasses is much more convenient to handle during preparation of the silage, and it is not necessary to neutralize molasses silage before use, as is usually the case with the AIV-fodder. A rise of 0·2 % in the fat content of milk was noted after feeding silage. It is possible, however, that the evaporation of organic acids and alcohols during the determination of 'dry matter', i.e. total weight minus water, was not duly taken into account. This error is often made in the determination of 'dry matter' in silage, which may thus appear to contain less potential foodstuff than is really the case. The products mentioned constitute a large part of the non-aqueous content and have a good food value. D-Lactic acid should be directly assimilable (Parnas, 1912) and L-lactic acid is excreted in the urine. Orla-Jensen, Orla-Jensen & Snog-Kjær (1948) pointed out that the calorific value of the sugar fermented is diminished only by one-third, through the formation of D-lactic acid, by two-thirds when DL-lactic acid is formed, but if L-lactic acid only is formed the nutritive value of the sugar is completely lost. Acetic acid and ethanol are of rather high food value and may be transformed into fat, which possibly explains the higher percentage of fat in milk after feeding ensiled pulp.

The fermentation during the ensiling process is known to consist mainly of a lactic acid fermentation, usually accompanied by a butyric acid fermentation when little fermentable sugar is present. Detailed studies of the progress of the fermentation and of the balance between fermented material and products of fermentation are rare in the literature. It was the aim of the present work to follow changes in composition and bacterial flora and to account for the losses of dry matter during the preparation of silage from sugar-beet pulp.

EXPERIMENTAL

Rubber bags which could hold 9–10 kg. pulp were used as containers. At one end each bag was fitted with a hose of thinner rubber through which it could be filled with pulp. After filling, the hose was rolled up and tightly tied with string so that gas or juice could not escape. At the other end of each bag a piece of rubber tubing was attached by means of glass tubing to a long rubber tube which led gas and juice to a reservoir where the amounts could be measured and samples taken for analysis. These long rubber tubes were led through the silage inside iron pipes to avoid distortions resulting in blockage. The reservoirs for press juice (seepage) were Wulff flasks which allowed collection of juice while gas was either permitted to escape or was led to a 5 l. flask filled with a saturated aqueous solution of NaCl, slightly acidified to diminish absorption of CO_2. The amount of liquid expelled by gas was measured, and samples of gas taken for analysis.

After weighing, the bags were placed in the middle of a concrete silo about 3 m. in depth and covered with tightly-packed pulp. This procedure was chosen in order to obtain the same conditions of pressure and temperature as those prevailing in an operating silo.

In order to discern differences in fermentation when little sugar was present (0·3–0·5 % of the pulp) as compared with the results when sufficient sugar for a pure lactic acid fermentation was present, pulp without any addition was compared with pulp + molasses (4 kg. molasses/100 kg. pulp). Before the addition of the molasses it was diluted with an equal weight of water.

The progress of fermentation was followed by withdrawing one bag of each type from the silo, initially every 2 or 3 days, and later less frequently. The contents of the bags were weighed, thoroughly mixed and samples taken, and at the same time the volumes of seepage in the Wulff flasks were measured and samples taken for analysis. When the silage itself was very moist, as was sometimes the case where no molasses was added, it was allowed to drain on a tray. It was considered that this 'drained-off juice' would have drained away from the silo and thus should be added to the seepage in the Wulff flasks to account for the total loss of material through seepage. Because the aim was to find out what happens during the fermentation proper, the amounts of the different substances in the seepage were added to the contents of the bags in the calculation of results, thus excluding factors other than the fermentation itself. The results of these calculations are shown in Figs. 3 and 4, while Table 5 shows the total losses of 'dry matter' due to fermentation and seepage.

Analytical methods

Bacteriological examination. Plating on Bacto-Tryptone glucose agar + 1 % sucrose with incubation for 5 days at 30° was used to identify betacocci (*Leuconostoc*) by the formation of dextran. After counting the plates, representative colonies were picked for later identification.

For the determination of butyric acid bacteria a series of dilutions ranging from 10^{-3} to 10^{-11} were inoculated into tubes of barley mash medium. The presence of butyric acid bacteria was detected by gas formation and the odour of butyric acid. The same procedure was used to determine the numbers of viable spores after pasteurization of the bacterial suspension at 80° for 10 min. This method was chosen because plating under anaerobic conditions or inoculation in deep agar tubes usually gave too small numbers.

Chemical analysis. As time did not permit an immediate complete analysis, 500 g. samples from the bags were given a preliminary drying at 70–80° and later analysed for: 'dry matter' (drying at 105°); ash (as sulphate); total N (Kjeldahl), protein being calculated by multiplication by 6·25; cellulose (Scharrer & Kürschner, 1931); pentosan (Kullgren & Tydén, 1929); galacturonic acid (Sloep, 1928).

Pentosans are determined in the method of Kullgren & Tydén (1929) as furfural after boiling and distillation with HCl. By this procedure galacturonic acid is also transformed into furfural + (CO_2 and H_2O). It was therefore considered necessary to correct the 'pentosans' for galacturonic acid, as estimated by the amount of CO_2 liberated by boiling with HCl (Sloep, 1928). In our hands this analysis did not give reproducible results; relative differences of *c.* 10 % were often found. In the analytical results shown later 'pentosan' therefore means the sum of pentosan proper + galacturonic acid. But from the analysis it may be concluded that the pentosans account for *c.* 40 % of the furfural-yielding substances, i.e. about 9 % of pulp 'dry matter'. As to losses of 'pentosan' it may be assumed that they apply to the real pentosans only, as galacturonic acid is hardly fermented.

Analyses of juice squeezed from silage. This was analysed for: *pH value* (glass electrode); *titration figure* (the figures obtained did not correlate well with the real amounts of acid, probably depending on the buffer content of the pulp); *sucrose* (Spengler, Tödt & Scheuer, 1936), after inversion with HCl, the values obtained of course include other substances capable of reducing Fehling's solution; *ammonia* (Folin, 1902). *Organic acids*; juice was acidified (H_2SO_4) and extracted with a measured volume of ethyl ether in a flask. According to the partition principle a certain percentage of each acid is taken up by the ether, from which the acids were separated by shaking with dilute NaOH. The alkaline aqueous phase was acidified, and volatile acids distilled off and determined by the method of Osburn, Wood & Werkman (1936). In the residue from this distillation, lactic acid was determined after Szeberěnyi's (1917) method (oxidation to acetic acid and subsequent distillation and titration). In this way the troublesome preparation of Schenk filtrates was avoided, and the results were reliable (detailed description of methods, Olsen, 1949). *Alcohols* were determined by a modification of the methods of van der Lek (1930) and of Enebo (1943) (see Olsen, 1949); by means of chromic-sulphuric acid, ethanol was oxidized to acetic acid and butanol to a mixture of acetic and butyric acids which were distilled off and analysed as above.

Seepage and drained-off juice were examined in the same way, and in addition dry matter, nitrogen, ash and pentosan were determined.

In the calculation of results account must be taken of the volatile substances which disappear during drying of samples, otherwise the determination of 'dry matter', i.e. total matter minus water, gives values which are too low. Experiment showed that when silage was dried at 105° about 50 % of the lactic, 90 % of the acetic, and 80 % of the butyric acids and the alcohols disappeared. By drying at 70–80°, 15 % of the lactic acid disappeared while the volatile acids and alcohols behaved as at 105°. When juices were dried at 105° all the acids disappeared completely. Thus it was necessary to know the amounts of these volatile substances before calculating the real percentage of 'dry-matter', i.e. total matter minus water.

When the volatile substances mentioned were summed up there still remained a considerable part of 'dry matter' to be accounted for. This consisted mainly of hexosans, i.e. the remainder of the hemicelluloses, the pentosans having been determined directly. The hexosans were calculated by subtracting the sum of the percentages of the substances determined from 100, the difference thus giving approximately the percentage of hexosan.

In experiments where molasses was added sucrose disappeared with surprising rapidity, and the amount could not all be accounted for by the acids formed. But when the sum (sucrose + hexosan) was calculated, a much better correlation was found between the decrease of this quantity and acid production. No explanation is offered for this, but as in a sense sucrose forms part of the hexosans it is included subsequently in 'hexosan', the results of the direct sucrose determinations being also shown separately.

After the conclusion of fermentation there was still some 'sucrose' in the silage. It amounted to 1½–2 % of the 'dry matter' in experiments without molasses and to *c.* 0·4 % when molasses had been added. Obviously we were here dealing with substances other than sugar capable of reducing Fehling's solution and which were largely formed during the fermentation, e.g. 2,3-butanediol and acetoin. The greater formation of these substances from pulp alone than from pulp + molasses undoubtedly depended on the fact that the fermentation without added molasses was more heterogeneous than in its presence.

RESULTS

Two series of experiments were made, and although the fermentation was more vigorous in the second than in the first, the sequence of events was so nearly the same that it is safe to regard them as identical. In the following description therefore, in the main, the second experiment is reported. Good agreement was found between the amounts of carbohydrate fermented and acids, alcohols and CO_2 formed.

The temperature in the silo itself was read daily from a maximum thermometer placed in an iron pipe inserted into the silage; readings were taken from 25 November to 31 January (Fig. 1). After an initial fall the temperature rose a few degrees, presumably as a consequence of heat generated by fermentation. In view of the vigour of the fermentation it might have been expected that the temperature would have risen more, but pulp containing *c.* 90 % water has a high heat capacity. After about 9 days the temperature fell steadily reaching only about 10° after 50 days, in spite of the low air temperature in January.

The gas production (Fig. 2), due to the activity of butyric acid bacteria, was evidently strongly suppressed by adding molasses. In the first experiment only CO_2 and H_2 were found, but in the second experiment unexplained trouble was encountered in determining H_2; the combustion was very slow and resulted in too high figures. Only traces of CO_2 were formed by the combustion, indicating probably traces of methane (3–5 % of total gas volume). Owing to these difficulties only the CO_2-content of the gases in the second experiment is shown in Table 1.

Table 1. *Carbon dioxide production from pulp with and without added molasses after different times of fermentation*

	Time after beginning experiment (days)				
Addition to pulp	5	8	12	18	12
	% CO_2 in evolved gas				
None	·	100	67·7	66·4	·
4 % molasses	63·5	72·5	72·3	72·1	75·5

In the second experiment the initial gas evolved was pure CO_2 originating from the lactic acid fermentation. Later, when only very small volumes of gas were being evolved, some other gas (H_2 ?) was also present. Without molasses increasing amounts of CO_2 were found, in agreement with the findings of Peterson & Fred (1932) in acetone-butanol fermentations.

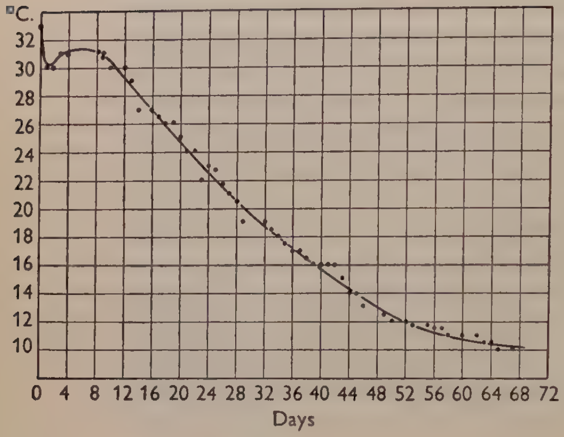

Fig. 1

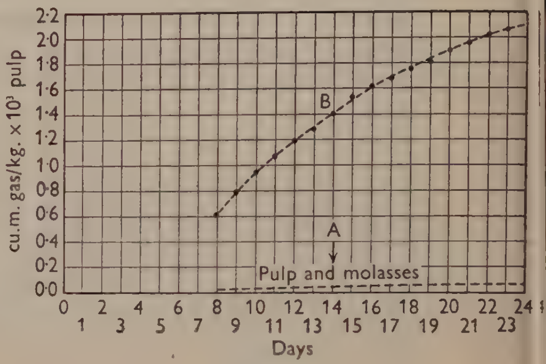

Fig. 2

Fig. 1. Temperature in the silo, 25 November – 31 January.

Fig. 2. Gas development from (A) pulp + molasses, (B) pulp alone.

Bacteriology of the ensilage

The percentage composition of the flora on the plates was estimated by the micro- and macroscopic appearance of the colonies, and a number of characteristic colonies were picked and identified. Lactic acid bacteria were predominant on the plates (butyric acid bacteria, of course, not being encountered on the aerobic plates). Sixty cultures were identified according to Orla-Jensen (1919), and were mainly rods, since the betacocci (*Leuconostoc*) usually give very characteristic mucoid colonies with sucrose present in the medium. All mucoid colonies were identified as *Betacoccus arabinosaceus* Orla-Jensen (*Leuconostoc mesenteroides* (Cienkowsky) van Tieghem).

The streptobacteria were an equal mixture of *Streptobacterium casei* and *S. plantarum* Orla-Jensen; no really clear distinction seems to exist between these two organisms. The betabacteria were *Betabacterium breve* Orla-Jensen. Yeasts were not encountered in any of the experiments.

All the strains of butyric acid bacteria isolated fermented lactose and lactate at pH 6·5 and 5·2. Van Beynum & Pette (1935–6) stated that their species *Clostridium tyrobutyricum* fermented lactate but not lactose, while their *Cl. saccharobutyricum* fermented lactose but not lactate. But many transition forms seem to exist; Richard (1948) found that ability to ferment these two substances was very variable within the same strain, and concluded that there was no reason for maintaining the species *Cl. tyrobutyricum* van Beynum & Pette. The strains here encountered may be identified as the common *Cl. butyricum* Prazmowsky.

The results of the bacteriological examinations are summarized in Table 2.

Table 2. *Results of bacteriological examination of samples of pulp taken during the course of the ensiling process*

(+ = pulp and molasses; − = pulp alone. Butyric acid bacteria counts were determined by finding greatest dilution inoculated into barley mash medium which gave butyric fermentation. Spores were determined after pasteurizing at 80° for 10 min.)

			Proportion of bacteria named (% of total)								Butyric acid bacteria/g. pulp			
Sample taken after (days)	Plate count following pulp		Beta-cocci		Strepto-bacteria		Beta-bacteria		Other types		Before pasteurizing		After pasteurizing	
	+	−	+	−	+	−	+	−	+	−	+	−	+ (spores)	−
0	10^7		80		.	.	.	.	Micrococci . Aerobic spore formers .	10 10	$<10^3$		.	.
									Micrococci					
3	10^{10}	10^{10}	70	75	15	8	.	.	15	17	$<10^3$	10^4	.	.
7	10^9	10^{10}	30	60	70	40	.	.	.	.	$<10^3$	10^7	$<10^3$	$<10^3$
12	10^{10}	10^{10}	20	50	80	50	.	.	.	.	$<10^3$	10^9	$<10^3$	10^4
15	10^{10}	10^{10}	20	30	80	50	.	20	.	.	$<10^3$	10^9	$<10^3$	$<10^3$
18	10^{10}	10^9	1	40	99	30	.	30	.	30	10^4	10^9	$<10^3$	$<10^3$
22	10^{10}	10^9	5	1	90	50	5	49	.	.	10^3	10^9	$<10^3$	$<10^3$
39	10^8	10^9	0	5	90	60	10	35	.	.	10^3	10^{10}	$<10^3$	$<10^3$
69	10^8	10^8	5	1	90	59	5	40	.	.	10^3	10^8	$<10^2$	10^4

The original pulp contained betacocci and aerobic spore formers in appreciable amounts; rod-shaped lactic acid bacteria were not detected in the dilutions used. The betacocci multiplied rapidly, while the bacilli seemed completely suppressed. After 3 days streptobacteria appeared in measurable numbers, increasing steadily in the following days while the betacocci decreased. After *c.* 20 days the very acid-resistant betabacteria appeared, a little earlier in samples without molasses than in those with.

A very pronounced difference existed between pulps with and without molasses with regard to the butyric acid bacteria. With added molasses less than 1000 organisms/g. were found (highest dilution tested 10^{-3}), but without molasses up to 10^{10} organisms/g. were found, the butyric acid bacteria here accounting for more than 50 % of the total population. After storage in the silo for 4–5 months, the numbers of butyric acid bacteria diminished, as a consequence of low pH values and exhaustion of food. Very few spores were formed, possibly because the acid reaction checked spore formation.

In connexion with the suppression of butyric acid bacteria in silage made with molasses, it is worth mentioning that undissociated lactic acid exerts a specific bactericidal effect. This means that at decreasing pH values a given amount of this acid will show an increasing toxic action not found with inorganic acids at the same pH. At higher pH values larger amounts of the acid are required to obtain the same effect (Richard, 1947). In the silage +

molasses the pH value drops more rapidly and much larger amounts of lactic acid are formed than in silage without molasses (see later), probably explaining the difference in numbers of butyric acid bacteria in the two kinds of silage.

Chemical changes during the silage process

The chemical analyses of the second experiment only will be recorded, since no important differences were found between the two sets of experiments. The results are given in Figs. 3 and 4. As the aim was to give a picture of the fermentation proper, the results include possible losses in seepage and drained-off juice (see later).

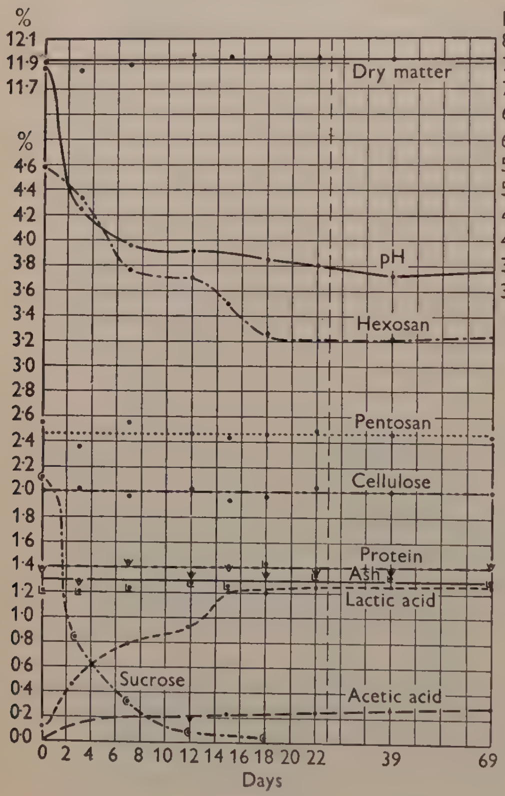

Fig. 3

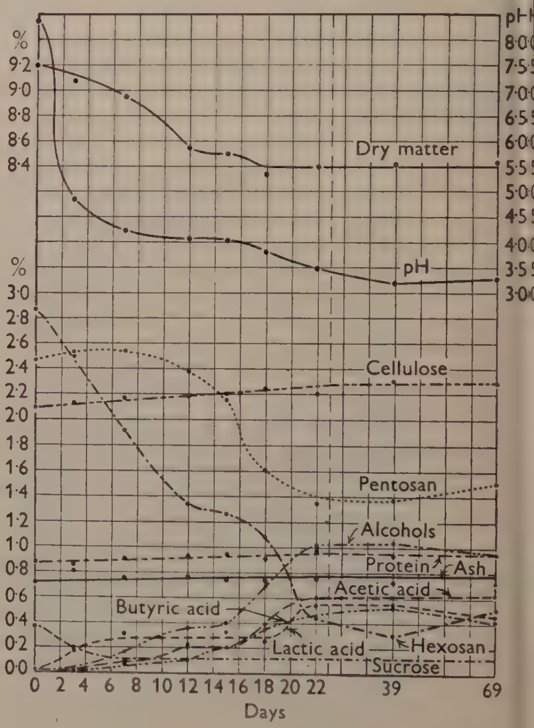

Fig. 4

Fig. 3. Experiment with pulp + molasses. pH values and percentage composition of the silage.

Fig. 4. Experiment with pulp alone. pH values and percentage composition of the silage.

Ensilage in presence of molasses. From the graph concerning the experiment with molasses the following conclusions may be drawn. A vigorous heterofermentative lactic acid fermentation begins almost at once. Only the 'hexosan' (sucrose + true hexosans) is subject to this fermentation, pentosan remaining almost unchanged during the whole experiment. Apparently only the sucrose part of the hexosan is attacked; at the start sucrose made up 30 % of 'hexosan' and at the end of the experiment this had diminished by 30 %. But the direct determinations of sucrose are not concordant with the other results (cf. p. 820).

Concomitant with acid formation, the pH value rapidly decreased, and after 6 days reached pH 4·0, then decreasing slowly to about 3·5. The dry-matter content was unchanged throughout the whole period; pentosan, cellulose, protein and ash remained unchanged since they are not attacked by the lactic acid bacteria. The amount of ammonia was also constant throughout the experiment (50–70 mg./kg. silage).

The curves show a close correlation between the disappearance of hexosan and the formation of lactic and acetic acids. It seems characteristic that between the 6th and 12th days the fermentation ceased and then began again at a rapid rate; this was observed in both series of experiments. The explanation is probably to be found in the transition of the bacterial flora from the less acid-producing betacocci to the streptobacteria. From Table 2 it is seen that in the same period the betacocci diminished in numbers and probably formed no more acid, while the streptobacteria slowly became predominant, finally being found in numbers so large that a vigorous homofermentative fermentation resulted. The amount of acetic acid remained practically constant, and butyric acid and alcohols were not found. The appearance of the silage had not changed; it was firm with a pleasant acid odour.

Ensilage in absence of added molasses. In experiments without molasses the results were quite different. Here only *c.* 0·4 % sucrose was present at the beginning and this amount was not equivalent to the amount of acids formed so that other sources of carbohydrate must have been used. From Fig. 4 it is seen that true hexosan as well as pentosan was fermented; *c.* 90 % of hexosan and 40 % of the furfural-yielding substances (i.e. all the true pentosan) were used.

In the initial stage there was a heterofermentative lactic acid fermentation until the sucrose had been used up, then butyric acid fermentation began, coinciding with the increase in numbers of butyric acid bacteria (Table 2). This resulted in the formation of butyric and acetic acids, alcohols, CO_2 and H_2; acetone was not detected. In Fig. 4 the 'alcohol curve' includes total alcohol which actually consisted, on the average, of a mixture of *c.* 80 % ethanol and 20 % butanol.

Lactic acid was almost constant from the 6th until the 17th day, when a sudden rise occurred. The quantities of other fermentation products remained constant from the 12th to the 16th day and then rose rapidly, coinciding well with the changes in pH value, hexosan and pentosan content. The pH value fell at first rapidly to about 4·2, remaining constant until about the 16th day when a new decrease occurred simultaneously with stronger acid formation (especially lactic acid).

The reason for this last decrease is probably to be found in a more vigorous breakdown of hemicelluloses making more pentose available. Betabacteria have a predilection for pentoses (arabinose); and by reference to Table 2 it is seen that, coincident with increase of lactic acid concentration, a considerable multiplication of betabacteria took place.

A loss of 8·3 % of the original dry matter was found, very nearly accounted for by CO_2 evolution. From Fig. 2 it is seen that gas development stopped when the loss of dry matter ceased. The CO_2 output however did not account completely for the loss of dry matter, the reason presumably being the formation of small amounts of volatile substances such as 2,3-butanediol. As previously mentioned, reducing substances other than sugar were estimated in the determination of 'sucrose'. Without molasses about 5 times as much of these substances was found as in presence of molasses; therefore probably the greater part of these reducing substances were formed during the butyric acid fermentation.

Cellulose, protein and ash remained unchanged and, corresponding with the loss in dry matter, constitute an increasing percentage of the latter and of the silage itself. Pentosan also remained constant, indicating that its fermentation did not begin immediately, but only when the sucrose and apparently some of the hexosan proper had been fermented.

Fermentation was less intense in the sample taken at 69 days than in that at 39 days, resulting in smaller amounts of fermentation products, a larger residue of hexosan, higher pH value, and less gas evolution.

The appearance of the silage made without added molasses was very unpleasant even at an early stage; the original beet slices were hardly recognizable, the whole being a rather homogeneous, slimy, very moist mass with an unpleasant sharp smell of butyric and acetic acids. The volume of the pulp was then almost halved. The same picture is very often observed under practical conditions. The altered appearance of the pulp is, of course, due to the breakdown of hemicelluloses which ordinarily maintain its texture.

Losses in seepage + drained-off juice

In the results reported the amounts of the different substances in seepage and drained-off juice have been included in the calculation so as to give a picture of the fermentation proper. In actual practice the losses in seepage play an important role.

From Table 3 it is evident that seepage from the silage with molasses was more concentrated than from silage without molasses. Drained-off juice (juice allowed to drain off after removal of silage from rubber bag) was only found in the experiment without molasses. This type of juice was almost as concentrated as the seepage from pulp + molasses, owing to the large amounts of acids and alcohols.

The amounts of juice and the losses of dry matter in it are shown in Table 4, where it is seen that the pulp has not retained all the liquid added with the molasses (4 % molasses + 4 % water). Hence, where molasses was added, small amounts of seepage occurred at an early stage, resulting in small losses of 'dry matter'. During ensilage variable amounts of seepage occurred; the loss of 'dry matter' at the end amounted to 2·2 % of the original.

Table 3. *Percentage composition of seepage and drained-off juice*

Component	Pulp + molasses In seepage (%)	Pulp alone In seepage (%)	Pulp alone In drained-off juice (%)
Dry matter	6·77	3·56	5·60
Ash	0·67	0·28	0·65
Protein	0·50	0·10	0·20
Pentosan	0·17	0·95	0·79
Hexosan	4·35	1·13	0·97
Lactic acid	0·91	0·70	0·55
Acetic acid	0·17	0·17	0·68
Butyric acid	·	0·03	0·56
Alcohol	·	0·20	1·20

Table 4. *Losses of 'dry matter' in seepage and drained-off juice from pulp alone or pulp and molasses*

	Pulp + molasses			Pulp alone			
		Loss of dry matter				Loss of dry matter	
After (days)	Seepage (l./kg. × 10^3)	(kg./kg. × 10^3)	Percentage of original	Seepage (l./kg. × 10^3)	Drainage (l./kg. × 10^3)	(kg./kg. × 10^3)	Percentage of original
3	0	0	0	0	0	0	0
7	16	1·1	0·9	0	0	0	0
12	16	1·1	0·9	0	0	0	0
15	18	1·2	1·0	21	0	0·8	0·9
18	0	0	0	71	0	2·5	2·7
22	33	2·2	1·8	75	68	6·5	7·1
39	12	0·8	0·7	120	70	8·2	8·9
69	39	2·6	2·2	56	35	3·9	4·2

With no added molasses, seepage did not occur until after 12 days, when it rapidly increased. After 22 days large amounts of drained-off juice were yielded and the resulting total losses in 'dry matter' reached *c.* 9 %. At 69 days less juice was found than at 39 days, agreeing well with the previously mentioned less vigorous fermentation in the last bag. It cannot, however, be said whether, under practical conditions in the silo itself, more of the drained-off juice would have appeared as seepage or would have been retained by the silage. The experiments of Steensberg (1945) showed that only small losses in dry matter were due to seepage. Under practical conditions the silage does not seem to release much water.

CONCLUSION

It has thus been possible to follow the fermentation processes in silage under practical conditions and to carry out many determinations during the course of the experiments, making chemical and bacteriological analyses.

Addition of molasses to sugar-beet pulp saved its 'dry-matter' content and diminished the number of butyric acid bacteria, thus giving a fodder rich in feeding units and suitable for milk production for cheese making. The addition of 40 kg. molasses/metric ton pulp gave a silage with 35 units more feeding value as compared with silage made without added molasses (see Table 5). Assuming that 1·3 kg. molasses has a feeding value of one feeding unit, the gain of feeding units in the molasses silage paid for the expense of the molasses used.

Table 5. *Losses in 'dry-matter' through fermentation and in seepage*

	Pulp + molasses		Pulp alone	
Losses	kg./kg. × 10^3	Percentage of original	kg./kg. × 10^3	Percentage of original
By fermentation	0	0	6·7	7·6
In seepage	2·6	2·2	8·2	8·9
Total	2·6	2·2	14·9	16·5

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The Assay of the Antibiotic Nisin by Means of a Reductase (Resazurin) test

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SUMMARY: The familiar resazurin test used in milk testing has been adapted to the estimation of nisin, using a fast-growing strain of *Streptococcus cremoris* as test organism. The reproducibility and accuracy of this test compares favourably with the many other assay methods for nisin which use *Strep. agalactiae* as test organism. Results may be obtained in less than an hour as compared with 15–48 hr. in the other tests and are a measure of bactericidal power only. Aseptic technique during the actual assay procedure is unnecessary. Methods were devised to ensure a suitable standard inoculum, which is imperative for this test. As little as 0·5 unit nisin/ml. may be estimated.

Inhibition of reductase activity and acid production by bacteria in milk have been used for the assay of penicillin and streptomycin. Reid & Brewer (1946) used *Bacillus subtilis* as test organism and added methylene blue as indicator system at the beginning of incubation. Approximate readings were possible after 4 hr. and final readings were taken after 18 hr. Sanchez & Lamensans (1947) used *Lactobacillus bulgaricus* as test organism. Their indicator system was a solution containing methyl red and methylene blue, drops of which were added every 30 min. to control tubes. The test could usually be completed within 2½ hr. Hirsch (1950) quotes a similar test as a rough guide for nisin activity, again using methylene blue as indicator, the test organism being a fast-growing strain of *Strep. cremoris* (1P5). The use of such a very fast-growing and sensitive organism (about four times as sensitive as *Strep. agalactiae*; Hirsch, 1950) makes it difficult to obtain a stable end-point; this is easily overshot if a reasonably accurate and therefore narrow dilution range is chosen. A graded response as obtainable with resazurin as indicator is therefore preferable to the all-or-nothing response of methylene blue. Moreover, methods must be devised to ensure a reasonably constant inoculum with regard to growth phase and number of bacteria. We have attempted to embody these requirements into a method which could be used on a routine scale with a minimum of labour, apparatus and experience.

MATERIALS

Cultures. Fast-growing strains of *Strep. cremoris* were used. The original N.I.R.D. strain '1P5' became somewhat sluggish and was eventually replaced by 'T17', a cheese-starter organism recently isolated from a culture from a Cheshire cheese factory. Doubtless many other strains would prove suitable. Subculture (0·1 %, v/v) was carried out every 24 hr. in the sterile milk medium given below. Incubation was at 30°.

Medium. Spray-dried skim milk (120 g.; Benger's Ltd., Coleraine) was reconstituted as a 12 % (w/v) solution in tap water. Volumes of 800 ml. were adjusted to pH 6·8 and sterilized by steaming for 30 min. on three successive days.

Resazurin solution. Five tablets weighing 0·12 g. each (Benger's Ltd.) were dissolved in 100 ml. glass-distilled water. Solutions were made up daily; they must be shielded from light.

Standards. A sample of partially purified nisin served as the master standard for all the work to be described. In collaboration with Dr Hirsch, according to the original definition of the nisin unit (Mattick & Hirsch, 1947) and using the original method of assay (dilution method; Mattick & Hirsch, 1947), 10,000 units/mg. have been attributed to this powder. Standards and subsidiary standard powders were stored at 12°. Fresh standard solutions containing, 10,000 units/ml. were prepared weekly in 0·05 N-HCl and stored at 12°.

Samples for assay and diluent. Powders were dissolved in 0·05 N-HCl and dilutions made in the sterile milk medium given above.

METHODS

Principle. The rate of reduction of resazurin in milk is indicative of the number of organisms present and was first used by Pesch & Simmert (1929) as a rapid and simple method for grading milk. Davis (1939) developed the test which was eventually made the basis of the official standard method in Great Britain (Ministry of Agriculture & Fisheries, 1943). Non-bacterial reducing systems interfere because of the extreme sensitivity of the dye which, on the other hand, ensures the great rapidity of the method.

Standard inoculation of sterile milk with a nisin-sensitive organism ensures a standard base-line activity modifiable by the action of the antibiotic. Reductase activity of other systems in the milk would raise this base-line, but not interfere with the test. The amount of antibiotic present is clearly indicated by the well-known colour changes of the dye; tubes containing sufficient nisin for the complete inhibition of the test organism show the unchanged blue colour of the control tubes containing the uninoculated sterile milk. Various possible methods were considered, and eventually the following was worked out.

PROCEDURE

Samples of nisin (5–15 mg.) are weighed out (torsion balance) and solutions made up to contain 1 mg./ml. Preliminary dilutions of these solutions are made in sterile milk such that 1 ml. of final dilution contains between 5 and 50 units of nisin. Graded volumes (0·1–1·0 ml. with normally 33 % differences; 10 % for very accurate work) of this dilution are accurately measured into successive tubes, followed by (on a routine scale) 4·6 ml. of the cold (2–4°) inoculated milk. A burette with a wide orifice is used for adding this bulk-inoculated milk, standardized as discussed below. Mixing is achieved by quick tipping of each tube, and the racks are then incubated for 15 min. at 30°. Not more than two racks (fifty tubes) are prepared by any one worker at one time because of the quick growth of the test organism. At least one standard series covering the range from 0·5 to 5 units/ml. is included with each set of tubes (two racks, fifty tubes). After 15 min. the racks are taken out of the bath (incubator) and 1·0 ml. of resazurin solution added to each tube. This addition must be carried out as quickly as possible, but need not be very accurate. Blow-out pipettes with very large orifices were used. Immediately after each addition the tube must be inverted by an assistant. The racks are then quickly returned to the bath for *at least* 5 min. to allow for colour development. Incubation is continued until a mauve to mauve-pink coloration is reached in the 1 unit/ml. standard tube. At this stage, the tubes are taken out and the colours noted. The following colour range (with the symbols used for scoring) is used: blue, B; mauve, M; mauve-pink, MP; pink-mauve, PM; strong pink, SP; light pink, LP; white, W. The B → M change gives the most reliable end-point. Blue corresponds for all practical purposes to 'all organisms killed' and is stable in the dark. Evaluation is therefore based on the first mauve tube (next to the last blue tube) as the end-point. The other tubes are, however, also taken into account, and matching may be carried out with the MP or PM tube as the end-point, although this is more difficult. Under the conditions of the test MP and PM tubes may change to P within 5 min. The P → W change is more gradual but reversible, and is therefore contra-indicated as an end-point. It may however, serve as a first useful approximation to the true value of the sample. 'W' should not occur in the standard range. The tubes were usually covered with glass caps or metal caps, but may be used uncovered. As the test is completed in 15–20 min. there is no need for aseptic precautions. Table 1 gives a protocol of an actual test.

Choice of inoculated milk for the test

Preliminary trials with the methylene-blue test (Hirsch, 1949) and the resazurin test gave extremely variable results. Not only is it difficult to ensure an optimum population every day, but the correct phase of growth for the action of nisin was at first unknown. Later experiments (see under 'Mode of action') confirmed first impressions that nisin acts on the actively growing organisms only. The following working scheme was devised to ensure reproducibility of population in the correct phase of growth for the purposes of our test, such that the end-point falls within the chosen test range and good colour differentiation is achieved between two neighbouring tubes. Each morning a 2·5, a 5 and a 10 % inoculum was made in sterile milk from a 24 hr. culture in milk and incubated for 1½ hr. at 30°, by which time it was in the log-phase. Further growth was then stopped by cooling in ice water and storing in the ice chest. The milk for the day's use was then chosen in the following manner:

(1) 5 ml. of each batch of milk were added to three test-tubes, 1*a* (10 % inoculum), 1*b* (5 % inoculum) and 1*c* (2·5 % inoculum), followed by 1 ml. of resazurin solution. The contents were quickly mixed by tipping and the colours allowed to develop for 5 min. at 30°. After this incubation the colours were quickly read and noted. (2) 0·2 ml. quantities of a 1/20 dilution in sterile milk of a standard solution containing 500 units nisin/ml. were added to tubes 2*a*, 2*b* and 2*c*. (3) 0·15 ml. quantities of the same 1/20 dilution were added to tubes 3*a*, 3*b* and 3*c*. (4) 5 ml. of the 10 % batch of milk were then added to

Table 1. *Protocol of a test*

(Symbols refer to the scoring of colour changes; see p. 832.)

Choice of size of inoculum for the given sample of milk

A solution of standard nisin (10,000 units/mg.) was made up to contain 1 mg./ml. Dilutions of this solution were made in the sterile milk medium and added in different volumes to the variously inoculated milk media.

	Volume of inoculum (%, v/v)		
	10 (*a*)	5 (*b*)	2·5 (*c*)
Composition of test	Colour of resazurin in test		
(1) Control; no nisin. 5 ml. of milk + 1 ml. resazurin; reading after 5 min. incubation	P	M	B
(2) 5 ml. of milk + 0·2 ml. of 1/400 dilution of nisin solution + 1 ml. resazurin; readings after 15 min. incubation	PM	M	B
(3) 5 ml. of milk + 0·15 ml. of 1/400 dilution of nisin solution + 1 ml. resazurin; readings after 15 min. incubation.	PM	MP	M

The inoculated milk of choice was therefore that containing 2·5 % (v/v) of inoculum, because the resazurin was not reduced in the control tube (no nisin) after 5 min. incubation, and this dilution of inoculum showed the most marked colour difference between the 0·15 and 0·20 ml. doses of the 1/400 dilution of nisin solution, namely B →M.

Test with chosen inoculum size (2·5 %, *v/v*) *in the given milk*

Dilutions of the samples refer to solutions containing 1 mg./ml.

	Volume of diluted nisin preparation (ml.)							Result (10^6 units/g.)
	0·1	0·15	0·22	0·35	0·5	0·75	1·0	
Samples (dilutions)	Colour of resazurin in test							
Standard (1/400)	PM	MP	M	B	B	B	B	10 (Assumed)
Sample 102.1 (1/40)	PM	MP	M	B	B	B	B	1
Gel 323 (1/4)	MP	M	B	B	B	B	B	0·147
Sample 100.1212 (1/800)	SP	PM	MP	M	B	B	B	12·6
Sample 87.1212 (1/800)	MP	M	B	B	B	B	B	29·4
Sample 90.2 (1/400)	LP	P	SP	PM	MP	M	B	2·95
Sample 91.2 (1/400)	W	W	W	LP	LP	LP	P	<2·2(?)
Standard (1/400)	PM	MP	M	B	B	B	B	10

tubes 2*a* and 3*a*, 5 ml. of the 5 % batch to tubes 2*b* and 3*b* and 5 ml. of the 2·5 % batch to tubes 2*c* and 3*c*. (5) After mixing the contents, all six tubes were incubated for 15 min. at 30°. (6) The tubes were then removed from the water-bath, 1 ml. of the resazurin solution added to each tube, the contents mixed once more and returned to the bath for another 5 min. The colours were then noted.

The milk of choice should be such that one of the tubes in (1) contains enough organisms to impart a pinkish tint while the next tube, containing milk inoculated with half the number of organisms, should not show any pink tints.

None of the tubes in (2) or (3) must go white. If that does happen, a 1/2 dilution in sterile milk may be resorted to and a fresh start made, although such a procedure has not always been satisfactory. The pair of tubes (2*a*–3*a* or 2*b*–3*b* or 2*c*–3*c*) showing the biggest difference in colour indicates the optimum growth phase and contains usually the batch of milk giving MP tint in (1). This milk is chosen for the day's use and maintained at 8–10°. It may, if necessary, be used also on the following day if kept in the refrigerator, but will certainly give slightly more reduction, i.e. shorter incubations will be necessary; it is always kept in the refrigerator except when actually adding it to the test solutions. Table 1 contains a protocol of an actual test.

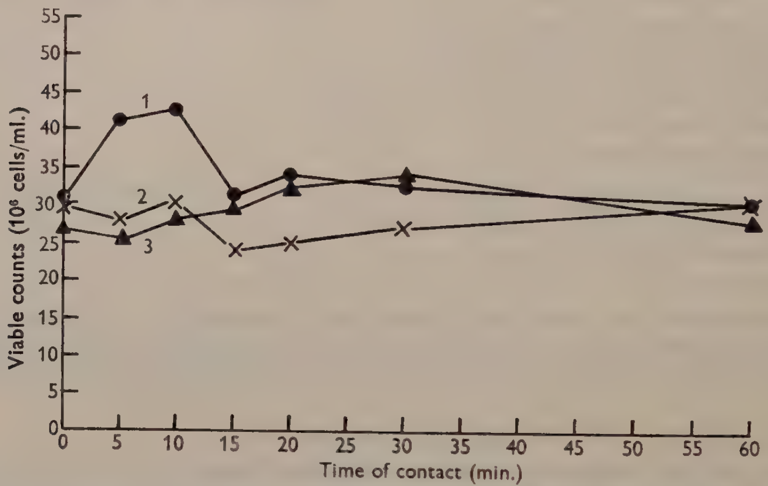

Fig. 1. The effect of 1 and 2 units nisin/ml. on *Strep. cremoris* in the lag-phase. Curve 1 (●—●), no nisin addition; curve 2 (×—×), 1 unit nisin/ml.; curve 3 (▲—▲), 2 units nisin/ml.

Mode of action of nisin

Viable counts were taken after leaving 1–2 units nisin/ml. in contact with: (*a*) freshly inoculated milk at 15° (Fig. 1); (*b*) freshly inoculated milk at 30°; (*c*) the same milk after 1½ hr. incubation at 30°. The organisms in (*a*) were thus in the lag-phase throughout the time of contact, while those in (*b*) were allowed to proceed into the log-phase during contact. Organisms in (*c*) started contact with nisin in the log-phase. Fig. 1 shows that, as expected, no significant decrease occurred in the viable counts during the lag-phase. These results were confirmed (Table 2) in a repeat of the experiment with a lower initial cell count in the presence of 2 units nisin/ml. In experiment (*b*), the total viable count in fact increased within 30 min. incubation at 30° from $4{\cdot}7 \times 10^6$ viable cells/ml. to 900×10^6 viable cells/ml. despite the presence of 10 units nisin/ml. After 2 hr. incubation, i.e. after about 30 min. contact of cells in the log-phase with nisin (intermediate counts not taken), the count dropped to < 10,000 cells/ml. In experiment (*c*), a rapid decrease in count occurred within 10 min. (Table 3). Mattick & Hirsch (1947) noted that *Strep. agalactiae* was killed within 10 min. The present tests therefore suggest that nisin exerts its

Table 2. *The effect of* 2 *units nisin/ml. on* Streptococcus cremoris *in the lag-phase*

Time of contact (min.)	Viable count (10^6 cells/ml.)
0	4·2
2	8·3
5	2·4
10	3·1
25	7·5
60	2·4

Table 3. *The effect of nisin on* Streptococcus cremoris *in the log-phase*

	Nisin concentration	
Time of contact (min.)	2 units/ml.	10 units/ml.
	Viable count (10^6 cells/ml.)	
0	8·3	9·5
2	5·7	—
5	3·2	—
10	0·8	0·2
20	—	0·01
25	0·7	—
30	—	0·03

bactericidal action on the actively-growing organisms only. Hirsch's finding (1950) that nisin delays the lag-phase, implying that there is some action on the resting bacteria, may have been a reflexion of the speed with which nisin kills organisms entering the log-phase. This would cause an apparent lag-phase.

Accuracy of the test

Powder 138 A was assayed 21 times giving a mean value of 25,800 units/mg. and a standard deviation for a single determination of 5740. The standard error was 2500 ($P=0·95$) or 10 %. Calculations of the standard error for a period of over a year of routine testing showed variations from between ±7 and ±20 % for six repeat assays, and with percentage differences of about 33 % between any two consecutive tubes (as illustrated in Table 1). Repeats carried out by one experienced tester only, usually yield absolutely reproducible results on the given (wide) scale. In order to obtain a measure of the possible accuracy of the test, percentage differences were adjusted to 10 % for a given powder which was assayed every day for 2 weeks. A factor of 10 % represents the limits for colour assessment. A standard deviation of 10·7 % and a standard error of 6·8 % ($P=0·95$) was obtained for the ten repeats (cf. Table 5, blend M).

Adaptation for use as a serial dilution test in micro-tubes

The possibility for a rough test in micro-tubes (75 × 8 mm.) was investigated, using a modified procedure. The resazurin was incorporated in the inoculated milk used in the serial dilutions. The desirable mauve end-point cannot be obtained under these conditions and we had to rely on the less stable pink → white end-point. However, in practice it was found that this end-point is very much more stable in micro-tubes (since there is a very small surface exposed to air) than in the 15 × 1·8 cm. test-tubes usually used; in fact, colours remained unaltered for up to 6 hr. at 30°. On incubation overnight the contents of some of the tubes solidified, giving an additional end-point.

The following procedure was worked out. (1) Dilutions are made in sterile milk. The samples are diluted to contain about 100 units/ml. (2) To 100 ml. inoculated milk (at 2–4°) 20 ml. of resazurin solution (prepared as in the macro test) are added. The mixture should exhibit a mauve tint. When the colour turns pink immediately, too many organisms are present and further dilution with sterile milk may be made. (3) 0·5 Ml. of the inoculated milk + resazurin is added to each tube. (4) 0·5 Ml. of the diluted sample is added to the 1st tube of each series and serial dilutions carried out. (5) Incubation at 30° is allowed to proceed for about 1 hr. until no further colour change is noticeable. (6) The colours are recorded. A standard is included with each set of twelve tests and the results are obtained by comparison with the standard as in the macro test.

The accuracy of this test is low, but reproducibility is good and it is a very convenient test for quick assessment. Samples of low unitage cannot, however, be assayed by this method, and no attention should be paid to the first three tubes, since acids and other interfering substances may not be sufficiently diluted out.

The effect of impurities on the assay

Hirsch (1950) stated that culture fluids containing nisin are very much more difficult to assay by the lag-phase method than are pure nisin preparations. It is obvious that such interference must be expected in a diffusion type of assay and was indeed found. When assaying penicillin solutions, it is possible to destroy the antibiotic by a penicillinase and then to test the effect of the medium alone on the test. This is not possible with nisin since so far a 'nisinase' has not been found. We therefore carried out the following experiment. Our standard solution of nisin (10,000 units/ml.) was diluted 20-fold with (*a*) 0·05 N-HCl; (*b*) culture medium before inoculation as used in the large-scale production of nisin; (*c*) culture fluid after nisin fermentation, assaying 600 units/ml.; (*d*) fermented culture fluid taken on an occasion when the fermentation had accidentally gone wrong and vigorous growth of producer organism had taken place without nisin formation. The assay result showed 500 units/ml. in cases (*a*), (*b*) and (*d*) and 1000 units/ml. in case (*c*), indicating that neither fermented nor sterile broth interfered with the test, nor the substances produced during the abnormal fermentation. It has been confirmed many times that the dilution, diffusion and lag-phase methods tend to give irregular results with crude culture fluids, while the resazurin method (macro test) usually gave results which were confirmed afterwards by the nisin yields obtained from the cultures.

Influence of acetone-HCl. During preliminary isolation experiments of nisin from fermented liquors, charcoal columns were eluted with 65 % (v/v) acetone in water containing also 2 % (v/v) of concentrated hydrochloric acid, the eluates collected and assayed before and after removal of acetone-HCl *in* *vacuo* (30°). The results are given in Table 4. With one exception (sample 18 E 2) the agreement was satisfactory. The nisin concentration of the eluates before removal of acetone was of the order of 200 units/ml. The conclusion that the 65 % acetone-HCl mixture did not interfere even at this low concentration of nisin seems justified.

Table 4. *The assay of nisin in the presence and absence of acetone-HCl (single assays)*

	Quantity of nisin found	
Sample	Before removal of acetone-HCl (units)	After removal of acetone-HCl (units)
14E	12,300	8,200
14H	9,400	9,250
18E1	37,200	34,100
18E2	38,400	94,000
18E3	10,000	10,600

DISCUSSION

The diversity of methods proposed for the estimation of nisin is a reflexion of the existing state of dissatisfaction. The *plate-count method* (Hirsch, 1950) is too cumbersome for routine testing and has very large limits of error. The '*dilution*' *method* (Mattick & Hirsch, 1947) has been used as a routine by us for several years. Unfortunately, under the conditions of the test, nisin is very readily and strongly adsorbed on to the glass walls (or to impurities sticking to the glass walls). As much as 2 units/ml. have been yielded up to sterile fresh broth placed in such vessels. Even boiling with detergents and alkaline or acid-oxidizing agents has not always been successful in removing the antibiotic, and the continuous use of new tubes is impracticable on a routine scale. The *lag-phase* method (Hirsch, 1950) has proved even worse on a routine scale. Its name is a misnomer: special precautions are taken to ensure that the test organism is in its most actively growing phase at the beginning of the test. It is also stated that finally 'the response due to the formation of resistant strains' was 'preferred to the lag-phase response'. In our opinion, however, this production of resistant strains constitutes only a modifying feature superimposed upon the usual direct bactericidal action of nisin on organisms in the log-phase (see Fig. 1). The test necessitates *p*H measurements within 0·1 of a pH unit, and the length of incubation has to be timed very accurately to obtain maximum differences of acidity between any two nisin concentrations. The response due to formation of resistant strains and that due to killing of the sensitive cells run in opposite directions, and the process of acid production is self-limiting; eventually all the cultures reach the same pH. In the *diffusion* method (Friedmann & Beach, 1950) diffuse zones were at times obtained with impure powders (similar interferences quoted for the lag-phase method) and the routine range is rather higher (100–1000 units) than in the other methods, although solutions down to 10 units/ml. could be assayed on a different range.

That method has nevertheless proved very convenient for routine purposes, although the limits of error are large unless many repeats are carried out; assay results cannot be obtained in less than 48 hr.

In all the methods just mentioned *Strep. agalactiae* is used as test organism. The use of the very fast-growing *Strep. cremoris* made the rapid *methylene blue* and *resazurin* tests possible. The short duration is justified owing to the very rapid bactericidal action of nisin on organisms in the log-phase. Thanks to the great sensitivity of the test organism and the low nisin concentration at which the test can therefore be carried out, impurities are readily diluted out in the highly buffered milk medium during the preliminary dilutions, resulting in less interference than in the other tests. 'Tube trouble', due to the adsorption of nisin on glass, has not been encountered, whether due to the different medium, the much shorter time of contact or for other reasons.

Table 5. *A comparison of assay results obtained with the diffusion, resazurin, dilution and lag-phase methods*

(Results for solids are expressed as $\times 10^{-3}$ units/g., those for 'brews' (specially indicated) in units/ml., and are given as arithmetic means ± standard error (in %) for $P=0\cdot95$. The number of single determinations is given in brackets. For the diffusion and lag-phase methods, these numbers are given as 10 × 5 and 10 × 3 respectively rather than as 50 and 30, since any one assay result in these tests is derived from 5 and 3 single estimations respectively.)

	Method of assay used					
	Diffusion		Resazurin		Dilution	Lag-phase
	Laboratory where tested					
Sample	Holmes Chapel	Loughborough	Holmes Chapel	Loughborough	Holmes Chapel	Loughborough
Blend M	7100 ± 10 (10 × 5)	5800 ± 12·6 (14 × 5)	6400 ± 25·6 (6)	6500 ± 10·2 (12) 6400* ± 6·8 (10)	650 ± 28 (4)	—
138 A	21400 ± 25·2 (13 × 5)	21500 ± 7 (14 × 5)	27000 ± 12·2 (10)	24000 ± 6·4 (10)	12400 ± 18·4 (5)	14500 ± 11 (18 × 3)
105	—	905 ± 10·4 (16 × 5)	—	677 ± 10·6 (12)	—	—
783	—	—	28500 ± 16 (6)	—	27500 ± 9 (6)	—
118	—	—	9700 ± 4 (10)	9900 ± 6 (10)	9200 ± 7 (9)	—
5.21 I	—	6100 ± 14 (7 × 5)	—	6100 ± 9 (6)	—	7000 ± 40 (6 × 3)
W71 A	—	2540 ± 13·6 (10 × 5)	—	—	—	2850 ± 30 (9 × 3)
Brew X	—	247 ± 14 (12 × 5)	—	231 ± 6·6 (12)	—	—
Brew 112	—	—	233 ± 12 (5)	—	194 × 46 (6)	—

* 10 % interval between consecutive tubes

Note. The only statistically significant discrepancy ('*t*'-test) occurs with powder 138 A, where the dilution and lag-phase methods gave lower results than the diffusion and resazurin methods, and has remained unexplained: neither unhydrolysed nor hydrolysed 138 A could be distinguished chromatographically from other powders of similar purity. The same test organism was used in the dilution, lag-phase and diffusion methods.

The test has proved suitable for measuring nisin in body fluids (Bavin, Falconer, Friedmann & Robson, 1951). The short duration of the test militates against interference due to the formation of resistant strains (necessarily forming a mixed population in all the other tests) or to the growth of contaminating bacteria. This speed has also been an advantage in stability testing since inactivations due to assay conditions are decreased to a minimum. It must, however, be emphasized that the strict standardization described is of paramount importance.

In Table 5 statistically analysed results for the four assay methods have been computed for nisin of varying degrees of impurity, and also crude culture fluids containing nisin. As far as the bulk of our assays over a period of about 2 years go, we feel justified in saying that no significant differences exist between the results obtained by the different methods, which thus all appear to measure the same entity. Apparent differences could usually be traced to different degrees of accuracy.

We have much pleasure in thanking: Dr Falconer for encouraging interest and useful criticisms; Dr Hirsch for interesting discussions; Mr A. S. Beach and staff of the Loughborough laboratories for carrying out some experiments relating to the mechanism of action of nisin and for allowing us to use data gained with the lag-phase and diffusion methods for our statistical analysis; Miss P. D. R. Smith and Miss P. Jewell for technical assistance; Messrs Fisons Ltd. for permission to publish.

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The Ecology of the Bacteria of Hobson's Brook, a Cambridgeshire Chalk Stream

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SUMMARY: The bacteria of the upper reaches of a chalk stream (Hobson's Brook) were studied as part of an inquiry into their association with ciliate protozoa. The bacteria studied were primarily those found in the current core at 2-month intervals as revealed by plating at 22°. The waters of the stream flow from chalk springs through arable land and are swift, cold, alkaline and usually rich in dissolved oxygen. These physical characters generally restricted the microflora to psychrophilic aerobes; but once, when turbulence was lost by damming of the water by a growth of *Nasturtium officinale*, micro-aerophilic and anaerobic bacteria tended to predominate. Near plants, especially blue-green algae, bacterial numbers were always much higher than in the current core.

The natural indigenous water flora was supplanted, after floods, continued heavy rain, or periods of drought, by a foreign microflora apparently coming from the adjacent soil. The latter flora was apparently modified by agricultural operations on the surrounding arable land and was partly recruited from the rhizospheres of cereal crops thereon.

A tributary stream contaminated by farmyard and domestic waste opens into the Brook but true *Escherichia coli* were only isolated from its mouth. It is suggested that the topography and turbulence of the Brook combine with protozoan activity to remove polluting bacteria rapidly.

Two strains of Gram-positive micrococci isolated from the Brook induced fission in ciliate protozoa. An unusual type of nitrogen-fixing bacterium was isolated on several occasions.

An investigation was made from October 1946 to January 1949 on the association between the ciliate protozoa and bacteria of Hobson's Brook, a short chalk stream near Cambridge. The inquiry was carried out as a study in microbiological ecology, and was in three parts: a study of the ciliate protozoa, of the bacteria, and of their relationship to the topography, physical factors and biota of the stream. This paper discusses the results obtained from the study of the bacteria. It must be emphasized that the study of the bacteria as such was undertaken only in relation to the whole object of the inquiry.

Topography. The stream flows from chalk springs (Nine Wells springs, Pl. 1, fig. 1) which lie *c.* 3 miles south of Cambridge. It is crossed by a main road which divides it into two stretches approximately equal in length: an upper rural reach surrounded by arable land, and a lower urban reach. Only the upper reach received detailed examination, as it is remote from human habitation (Fig. 1). Certain sharp bends made it possible to divide this reach into four arbitrary Zones (Fig. 2):

Zone I was from the springs to a railway culvert 100 yards downstream; a typical spring was chosen to represent this zone.

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* This investigation was carried out immediately before joining the Service, at the University School of Botany, Cambridge.

Zone II extended below the culvert. It had a chalk floor followed by a long stretch of gravel sloping down over the 46 ft. contour. The water over the gravel was shallow and swift (Pl. 1, fig. 2).

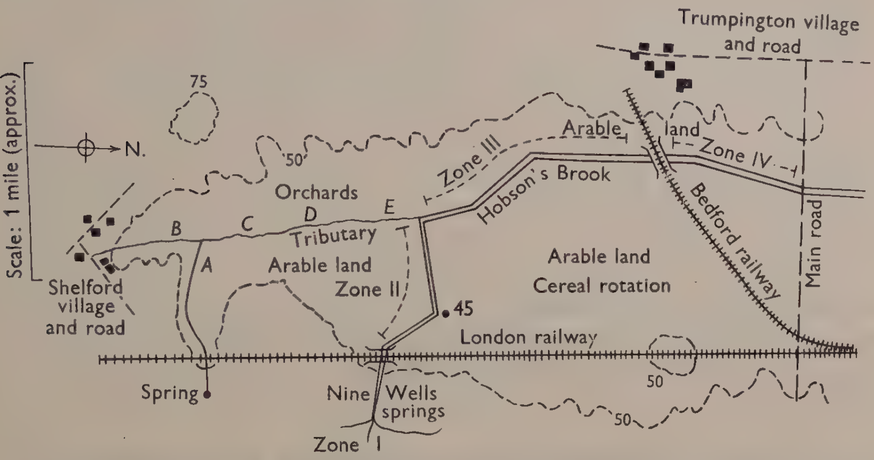

Fig. 1.* The topography of the stream and its tributary, showing the contours (in broken line, ----), the main roads (heavy broken line, ———) and the railways and bridges. The arbitrary zones are marked by Roman capitals and the sampling points in the tributary by the letters *A–E*. The figure 45 in the centre of Zone II is a surveyors' spot level and the water runs swiftly down from this point into Zone III. A second spot level, marked 38 (not shown in the figure), is marked approximately 1200 yd. downstream from the main road which terminates Zone IV and some 500 yd. from the end of the stream. The fall is therefore only 7 ft. from the centre of Zone II to near the point where the stream terminates. Nevertheless, there is always a marked current in the stream, especially in the upper reaches, in contrast to the tributary whose flow is negligible.

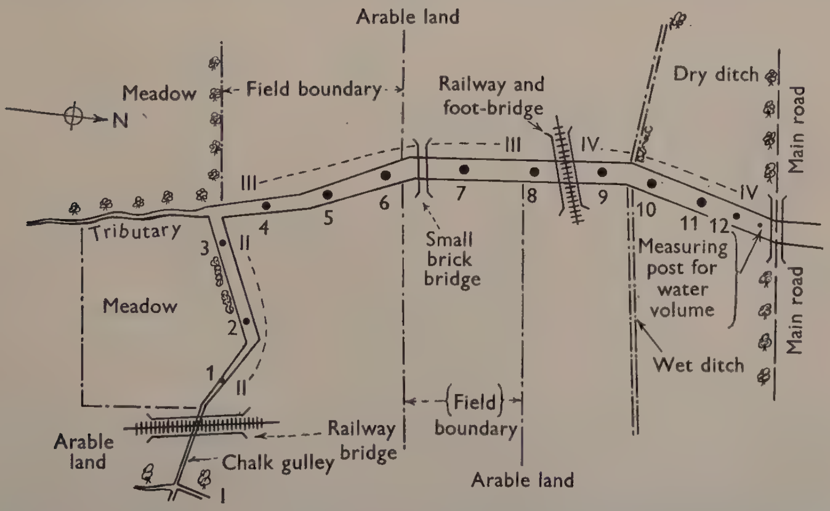

Fig. 2.* The rural reach of the stream showing the Zones (in Roman capitals) and the 12 sampling stations (in Arabic numerals). Trees and hedges exist only where shown; all the bridges pass over the stream allowing a free run of water.

* These maps are based upon the Ordnance Survey Map with the sanction of the Controller of H.M. Stationery Office.

Zone III ran from the end of Zone II, to a point where the stream is crossed by a second railway line. Its floor was largely composed of earth and chalk, with two patches of gravel (Pl. 1, fig. 3).

Zone IV ran from below the railway bridge to the main road which terminated the reach; it had a floor of deposited silt over chalk and chalk mud (Pl. 1, fig. 4).

Physical characters. The waters of the Brook are cold and alkaline. The upper reaches (Zones I to II and the upper third of Zone III) had a more uniform temperature than the lower reaches (the lower third of Zone III and Zone IV) which were warmer in summer and colder in winter. The typical spring was rheocrene (cf. Carpenter, 1928, p. 225) in character with neutral or slightly acid waters. The reaction in Zone II tended to be almost neutral; through Zones III and IV the water became increasingly alkaline. Some random tests indicated that the winter waters were virtually saturated by oxygen, but as the year progressed the content diminished except in the rapid gravel reach (Zone II), as shown in Fig. 3. The characters of the Zones are summarized in Table 1.

Table 1. *The physical features of the Zones of Hobson's Brook*

The Zones are arbitrary but imposed by the topography and rate of flow in each. Fig. 1 shows the surveyors' spot level at the beginning of Zone II and the contours which are responsible for the flow of water in Zone III. The output of the springs was measured at the railway bridge between Zones I and II, immediately below the chalk gulley (Fig. 2). The linear rate of flow was measured in Zone II between sampling points 2 and 3, and in Zones III and IV opposite the sampling points 5 and 11 (Fig. 2). The floor between sampling points 2 and 3 is gravel, and there are scattered gravel patches between points 6 and 7, and about point 9. Elsewhere the floor is chalky, covered by some depth of mud between sampling points 9–12. The bridge between sampling points 8 and 9 (Fig. 2) is double, carrying not only a railway but a footpath. None of the bridges normally causes any obstruction to the rate of flow.

Zone: ...	I	II	III	IV
Length (yd.):	—	486	868	694
Rate of flow in linear ft./sec.:	(Output 1·5–2·2 cu.ft./sec.)	2	1	0·2
Temperature (° C.)				
Maximum:	10	11	14	15
Minimum:	8	8	7	5
Average:	10	12	12	12
pH value:	6·6–7·0	7·0–7·2	7·4–7·6	Up to 7·8

METHODS

Sampling and counting

The current core only was sampled throughout the investigation. Samples for routine examination were taken from clear water in mid-stream, and at the spring from the jet of water issuing from fissures in the chalk. No samples were taken from the bottom mud, for the stream was so swift that it was considered that at any given time the most representative microflora would be carried along by its current, whereas in the mud the flora would tend to be specialized by sedimentation and anaerobiosis.

The samples fell into several series:

Series (*i*). As routine, the current core was sampled every 2 months in each zone. In Zone I, only a single spring was selected for sampling. In Zones II-IV, samples were taken at the points indicated by the Arabic numerals in Fig. 2.

Series (*ii*). In relation to the numbers and food habits of the associated ciliate protozoa, 100 counts of bacterial numbers at 22° were made in the early summer of 1947. These samples were taken at the twelve stations (1–12) shown in Fig. 2.

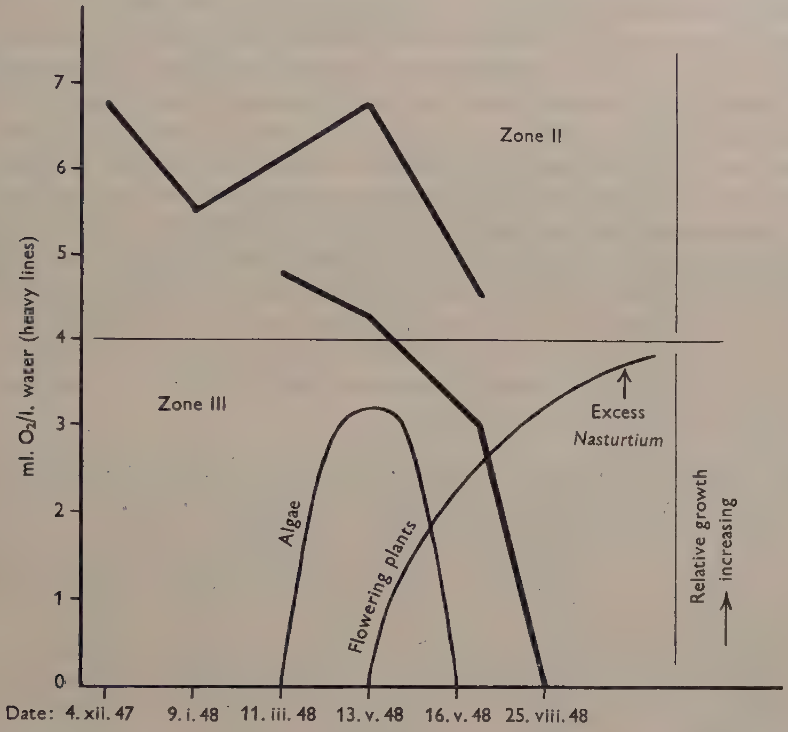

Fig. 3. Dissolved oxygen in the Brook during 1947–8, estimated by a colorimetric modification of the Winkler technique.

Series (*iii*). Also in relation to the numbers and food habits of the protozoa, extra samples were taken in the middle third of Zone III in the summer of 1948, twice a week at five fixed points between stations 6 and 8. In these samples, only the morphology of the bacteria, as shown by Gram-staining, was determined.

Series (*iv*). In addition, random samples were taken throughout the investigation so far as possible in relation to the twelve fixed points shown in Fig. 2, to examine the microflora associated with eddies or fixed objects such as plants.

Complete bacteriological examination, as described below, was given only to samples from *Series* (*i*) and (*iv*). Samples for bacteriological examination were taken in 8 oz. 'medical flat' bottles (capacity approximately 240 ml.) which were thoroughly rinsed after use in cold tap water followed by distilled water.

No detergents were used to clean the bottles, as it was felt important not to introduce any chemicals. The bottles were plugged by cotton-wool covered with Kraft paper and sterilized by dry heat. Before use the cotton-wool was replaced by a sterile rubber stopper, the paper cap being replaced. Samples were taken when working upstream, to avoid contamination by stirred-up mud. The bottles were filled to capacity and after sampling was completed the rubber bung was inserted, recapped with Kraft paper and the temperature of the stream taken. Samples were examined without delay after they were taken, for the bacterial numbers and species quickly changed.

Viable counts were determined as follows. The bottle was well shaken and dilutions made in sterile distilled water. Immediately after the dilution had been made the pH of the sample was determined colorimetrically. Circumstances limited the writer to one procedure, and the media and times of incubation chosen follow the regulations laid down by the Ministry of Agriculture for the bacteriological examination of farm water supplies. The standard medium used throughout for plating and subculture was a Yeastrel-peptone agar containing 0·1 % (v/v) whole milk, clarified by filtration. At the first few routine tests of the current core, duplicate plates were incubated at 22° and 37° respectively, but (except occasionally in Zone IV) a greater number of bacteria developed at 22° so that incubation at 37° was not used as a routine procedure (Table 2). Plates were incubated for 4 days at 22° (or 37°) before colonies were counted.

Table 2. *Comparative viable counts at 22° and 37° in the respective Zones during the first three routine samplings of the current core*

	Temp. of count	Count (thousands/ml.)		
Zone	(° C.)	October	December	February
I	37	1	1	0
	22	10	30	1
II	37	2	9	2
	22	22	8	19
III	37	2	4	8
	22	26	10	25
IV	37	216	16	73
	22	262	16	14

Isolation and media

Colonies picked off were purified where necessary after examination of streak cultures in Gram-stained preparations. When pure, they were inoculated into bouillon. Motility was ascertained by examining 12 hr. cultures in peptone water; alternatively a drop of the peptone water culture was allowed to diffuse over the surface of nutrient agar for 12 hr. when the edge of the drop was cut out and the organisms allowed to diffuse in watch-glasses containing peptone water or sterile saline.

After the morphology of an organism had been ascertained it was tested as a routine in nine media: glucose broth (for the Voges-Proskauer and Methyl

Red reactions); nitrate broth (0·1 % potassium nitrate in peptone water); bouillon or nutrient (Lab-Lemco) broth; nutrient gelatin (made with Lab-Lemco broth); litmus milk (skimmed-milk powder); glucose broth (1 % glucose in peptone water with 0·25 % (v/v) of 1 % aqueous solution of neutral red as indicator); inorganic (peptone free) sugar media containing 1 % salicin, lactose or maltose with bromcresol purple as indicator.

Gram-negative rods which gave a positive V.P. or M.R. reaction were further tested in a citrate medium (virtually Koser's, except that citric acid was used instead of sodium citrate following the formula of the Ministry of Health Report no. 71, 1939). All media were adjusted to pH 7·2–7·4 and tested for sterility by incubation at 22° and 37°.

When spore-formers dominated the microflora, a test was made to detect anaerobic strains. A few drops of a 24–48 hr. bouillon culture were added to a sterile tube of whole milk sealed with sterile liquid paraffin. The whole was then pasteurized at 80° for 30 min., allowed to cool and incubated at 37° for 2 days. Stormy fermentation in the curd and the production of a clear whey indicated the presence of *Clostridium welchii* which, as a typical form, was regarded as an indicator.

RESULTS

The quantitative aspect

Table 3 gives the total bacterial numbers obtained in the current core of each Zone from the samples of *Series* (*i*). Three things appear from this table: (*a*) The general level of the counts, even in Zone I, was rather higher than that usual in the waters of springs. (*b*) There was a general increase in bacterial numbers downstream to Zones III and IV, especially in the latter. This was associated with increase in alkalinity, increase of organic content in the water, and increased opportunity for contamination. (*c*) There was a strong suggestion that the bacteria were more numerous in 1948 than in 1947. This may have been related to the high rainfall in 1948 (as will shortly be seen) which is likely

Table 3. *Number of viable bacteria at* 22° *obtained from the samples of Series* (*i*), *from October* 1946 *to December* 1948

		Number of viable bacteria (10^3/ml.)					
Zone	Year	February	April	June	August	October	December
I	1946	0·5	.	.	.	5	15
	1947	0·5	1	2	2	14	40
	1948	0·5	8·5	60	2	0·5	228
II	1946	.	.	.	.	11	4
	1947	9·5	3	3	14·5	22	200
	1948	2	1	8·5	8	14·5	4·5
III	1946	.	.	.	.	13	5
	1947	12·5	3	16	4·5	20	215
	1948	3	12·5	24	363	12·5	6·5
IV	1946	.	.	.	.	131	5
	1947	7	193	18	50	60	.
	1948	11	9	16·5	341	4·5	49

to have resulted in increased numbers of soil organisms being washed into the stream. *Topley & Wilson's Principles* (1946, p. 2018) quotes similar observations.

The effect of excessive rainfall is illustrated by Fig. 4, which shows the relation between the bacterial numbers in Zone IV and the water volume during the thaw that followed the severe winter of 1947. It is plain from Fig. 4 that as the water-level increased so did the numbers of bacteria in the water. It is presumed that this was due to soil washed into the stream, for the water was very turbid and contained organisms of soil type, e.g. *Pseudomonas fluorescens* and *Escherichia coli* irregulars (compare Table 4 below). Such effects would be more noticeable in the lower Zones. No attempt was made to relate the bacteria of the water at the springs to rainfall, as this water was filtered through the chalk.

Table 4. *Bacteria isolated from the current core. (The numbers in brackets indicate the Zones where the bacteria were generally found)*

Members of all these species occurred irregularly in all Zones at all seasons of the year (except for *Chromobacterium violaceum* var., and *Flavobacterium* spp.—see p. 847). Although in general distribution was irregular, certain species were more common in certain months than at other times, as indicated.

Identified

Aerobacter aerogenes var. (II.) Isolated once, in December 1946.
Escherichia coli Intermediate Type 2. (II.) Isolated in April and August 1948.
Esch. cloacae var. *ceratophyllum*. (III.) Isolated once, in October 1946.
Bacillus mycoides. (I, IV.) Isolated sporadically: v. common. October 1946, April 1949.
Chromobacterium prodigiosum. (IV.) Isolated in October 1946 only.
C. violaceum var. (III, IV.) Isolated in February and April 1947 only.
Pseudomonas fluorescens non-liquefaciens. (III). October 1947 and February 1948 only.
P. fluorescens-liquefaciens. (III.) In June 1948 only.

Unidentified

Achromobacterium spp.? (I II, III, IV.) Isolated in October, December 1948.
Actinomyces spp. (I, II, III, IV.) Most common in the spring of 1948.
Bacillus spp. (I, II, III, IV.) Most common in the late summer of 1947, and the spring of 1949.
Bacterium spp. (I, II, III, IV.) Most common in June 1947 and April 1948.
Bacterium (chromogenic) spp. (III, IV.) Isolated in February and June 1947.
Esch. coli var. (I, II, III, IV.) Most common in April, August 1947, June 1948.
Corynebacterium spp. (I, II, III, IV.) Most common in December 1946, and August and October 1948.
Chromobacterium (*Flavobacterium*) spp. (I, II, III, IV). Dominated the bacterial flora from December 1946 to February 1947; not subsequently isolated.
Lactobacillus spp. (III.) Common in December 1948.
Micrococcus spp. (I, II, III, IV.) Generally speaking commonest in the springs in winter, in spring and summer elsewhere.
Micrococcus (chromogenic) spp. (I, II, III, IV.) Irregular in occurrence.
Pseudomonas spp. (I, II, III, IV.) Commonest in June 1947, February 1948, April 1949.

Variation of temperature usually causes an inverse variation of bacterial numbers in water of low organic content, and a direct variation in waters of high organic content (e.g. *Topley & Wilson's Principles*, 1946, p. 2018). On this basis it might have been expected that counts in Zone II would be lower in summer than winter, and in Zone IV higher in summer than in winter for, as compared with other zones, Zone IV had a high silt content. The data of Fig. 5, however, show no such relations. There is some suggestion of an increase of bacterial numbers in winter but it is not clearly related to the low temperatures. One would conclude that in this stream the influence of temperature is insignificant compared with that of rainfall.

The qualitative aspect

Early in the investigation it was found that a division could be made into an indigenous flora of water bacteria and a foreign flora apparently derived from soil. The natural water flora comprised species of *Bacterium*, *Pseudomonas*, *Flavobacterium*, *Chromobacterium* and perhaps *Micrococcus*. The foreign flora consisted of Gram-positive spore-forming rods such as *Bacillus mycoides*, *B. subtilis*; Gram-positive non-sporing rods, such as *Corynebacterium* and *Lactobacillus* spp., together with species of *Actinomyces*; and Gram-negative rods such as *Aerobacter aerogenes* and atypical *Esch. coli*.

The distinction between the natural water microflora and that from external sources is somewhat arbitrary, but it is drawn partly from Taylor (1942), who stated that as compared with soil the chief difference is the absence from water of the genera *Mycobacterium* and *Corynebacterium* and of *Bact. globiforme* Conn, and is also based upon the typical members of the soil microflora as given by Waksman (1928). It was found, too, that in general the members of the natural water flora had little biochemical activity (cf. Taylor, 1942), whereas the activities of the foreign flora were more pronounced.

In addition, there was a large group of obscure Gram-negative rods with feeble biochemical activities whose exact nature was not determined and which were much in evidence in 1948. The only organism found to fix atmospheric nitrogen was a new form, isolated from the Brook and also from the Cam and a neighbouring stream the Bourn Brook (Gray & Smith, 1950).

A list of the types isolated is given in Table 4. Only in relatively few instances did the organism appear to have been previously described. On the basis of morphology, cultural and physiological characteristics it was possible to get close to naming species of *Flavobacterium* and *Pseudomonas*. Some of the obscure Gram-negative rods (non-motile or motile with peritrichous flagella) appeared to be achromobacteria, whilst others produced pigment (red, orange or dirty white to yellow); Gram-negative rods producing pigment are stated by Taylor (1942) to be characteristic water bacteria. Cultural characters of some of these Gram-negative rods are given in Table 5.

The natural bacterial flora

Although temperature had no obvious effect upon the number of bacteria, it had an effect on the nature of the bacterial flora, and so indirectly upon bacterial numbers. The water of the Brook is cold and its bacterial flora is largely composed of psychrophilic bacteria (optimum temperatures below 20°; Thomas & Sekhar, 1946). When during the severe winter of 1946–7 the Brook temperatures fell to an average of 6°, there appeared strains of *Chromobacterium* *violaceum* (isolated by Schmelch (1888) from melting glacial snow and from the Thames in winter by Ward (1898)), followed almost at once by a great efflorescence of flavobacteria which dominated the flora everywhere in the stream. *Chr. violaceum* did not appear in February 1948 when the Brook

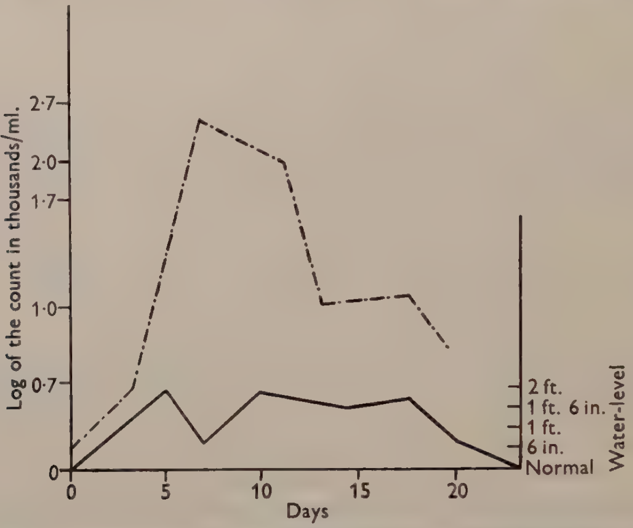

Fig. 4. Viable counts at 22° and water volume in Zone IV during the great thaw of March 1947.

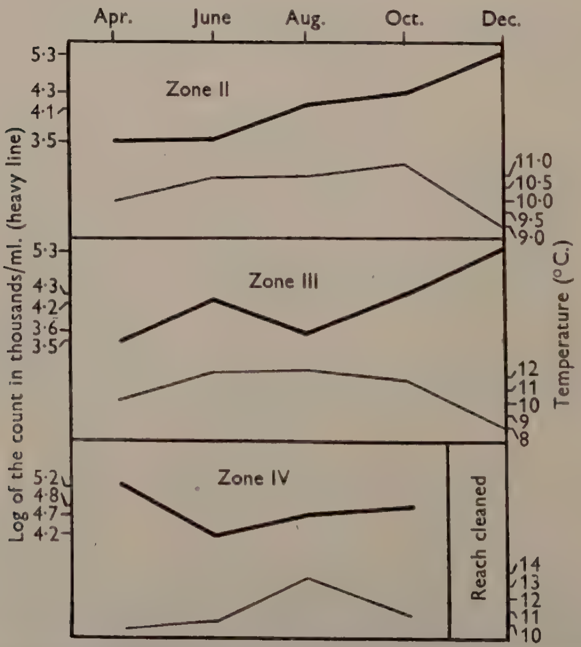

Fig. 5. Temperature and viable counts (bimonthly sampling) in the various Zones during the summer and winter of 1947.

Table 5. *The morphology and biochemical reactions of some of the Gram-negative rods of unknown origin*

No.	Morphology	Colonial appearance on nutrient agar	Gelatin liquefaction	Production of acid (A) or gas (G)			Growth in		Nitrate reduced to nitrite	Ammonia production
				Maltose	Lactose	Glucose	Broth	Litmus milk		
1	Non-motile rod	Flat, grey confluent	Infundibular, turbid ring	A (feeble)	A	A	Heavy turbidity ring	Reduced, alkaline	+	–
2	Non-motile cocco-bacillus	White, flat, pin point	Negative	–	–	A	Pellicle	Alkaline, no reduction	+	trace
3	Non-motile slender rod	Circular, lemon, butyrous	Crateriform	A	A	–	Light growth, granular deposit	Acid, reduced, peptonized	+	–
4	Non-motile rod	Round, grey, flat	Stratiform	–	A (feeble)	–	Clear supernatant, granular deposit ring	No change	++	+
5	Motile slender rod	Grey, irregular	Slow, napiform	–	–	–	Light turbidity	Slow alkaline reduction	–	trace
6	Motile rod	Flat, white, round	Stratiform	A	–	AG	Light turbidity, pellicle	Acid, peptonized, reduced	+	–
7	Motile small rod	Grey, irregular	Stratiform	–	–	A (pellicle)	Little growth	Slow alkaline reduction	–	+
8	Motile small rod	Diffuse, arborescent	Stratiform	–	–	A	Thick pellicle, fringed	Reduced, alkaline, digestion	–	+
9	Motile cocco-bacillus	Flat, white, round	Stratiform	A (feeble)	–	A (pellicle)	Diffuse turbidity, oily pellicle	Alkaline, reduced	–	+
10	Motile slender rod	White, arborescent	Negative	–	–	–	Clear supernatant, heavy deposit	No change	trace	–
11	Cocco-bacillus motility poor	Pale, semi-translucent	Filiform	AG	AG	AG	Diffuse turbidity, fringed pellicle	Not observed	++	–
12	Motile small rod	Amoeboid, white	Stratiform	–	AG	AG	Heavy turbidity	Reduced, clot, acid	+	–
13	Non-motile rod	Circular, yellow butyrous	Slow, stratiform	A (feeble)	A (feeble)	– –	Light turbidity, granular deposit	No change	+	–
14	Motile slender rod	Circular, yellow, butyrous	Stratiform	A	A	–	Diffuse heavy turbidity	Acid, peptonized, reduced	+	+
15	Motile slender rod	Circular, crenated edge, orange	Filiform	A	A	–	Turbidity, granular deposit	Acid, peptonized, reduced	+	–
16	Motile rod	Flat, round, red	Crateriform	AG	–	AG	Turbid deposit	Slow acid, reduction	+	–

The V.P. reaction was negative in all cases. The M.R. reaction was positive only with nos. 11, 12 and 16. Salicin was fermented only by no. 11 (acid only), and no. 12 (acid + gas).

temperature was 8–9° nor did flavobacteria except in isolated instances. During the bimonthly examinations carried out from 1 October 1946 to 1 October 1948, 48 strains of bacteria from the springs of Zone I, 47 strains from Zone II, 47 strains from Zone III, and 50 strains from Zone IV received detailed examinations, and it was generally found that those organisms regarded as true water forms (e.g. achromobacteria, micrococci? and chromogenic bacteria) tended to occur late in the year or in January and February, when the temperatures were lower.

As regards the *Micrococcus* spp. it was doubtful whether or not they were true water forms. All were Gram-positive but none was exactly identified. Though they were common in the Brook, they were not rare in the soil of the banks. When the cultural characteristics of four strains isolated from Zone III were compared with four strains isolated from the soil of the banks of Zone III, the first four strains of soil micrococci all liquefied gelatin rapidly, whereas those from the brook did not (Table 6). Subsequently, however, soil strains were isolated from the banks which did not liquefy gelatin, and water strains which did liquefy it within a few days. Certain of these strains were subcultured during nearly 2 years and did not change their morphology, showing that they were true micrococci and not pleomorphic strains of *Bact. globiforme* Conn. Further, filtrates of two of these strains induced binary fission in ciliate protozoa; Sonneborn (1932) found that chain formation in *Colpidium colpoda* was induced by a strain of *Micrococcus sensibilis*.

Table 6. *Cultural reactions of micrococci isolated in December* 1947 *from the current core of Hobson's Brook, and from soil within* 10 *yards of the banks*

Strain	Gelatin stab	Formation of acid (A) or gas (G)			Litmus milk	Nitrate reduced to nitrite
		Salicin	Lactose	Maltose		
1	Infund.	A	—	—	Peptonized reduced	++
2	Infund.	A	A	A	Peptonized reduced	–
3	Stratif.	—	—	A	Peptonized reduced	++
4	Stratif.	—	—	—	Peptonized reduced	++
5	Not liquef.	—	—	—	Slightly alkaline	+
6	Filiform growth, no liquef.	—	—	—	No change	—
7	Not liquef.	—	—	Feeble A (glucose)	No change	—
8	Filiform, no liquef.	—	—	—	Slightly alkaline	—

Only strain 8 produced ammonia.

The foreign bacterial flora

The foreign bacterial flora, presumed to come from the soil, appeared after rain and also after prolonged periods of hot dry weather when soil continually crumbled into the water. But for this foreign flora to dominate the microflora of the Brook, it was necessary for such climatic conditions to act over a considerable period of time. When earth was constantly washed in during prolonged showery weather, or continually crumbled into the stream during droughts, the effect was more lasting than that of a sudden flood which dispersed as quickly. Considerable floods appeared, for example, in March 1947 and filled the stream with soil, but by April of that year when the water subsided only a few soil bacteria were present (e.g. *Actinomyces* sp. in Zone III, unidentified spore-formers in Zone IV). By contrast, in August 1947 after a hot dry summer, when soil had been constantly observed to fall into the stream, especially under the concussion of agricultural machinery during harvesting, soil bacteria dominated the flora, *Esch. coli* irregulars and *B. subtilis* being isolated from all Zones.

The character of the foreign flora entering the Brook was considered to be largely determined by the agricultural operations on the surrounding land. Severin (1895; quoted by Waksman, 1927) found rods to predominate in freshly-ploughed soil but within 2 weeks micrococci predominated. Table 7 shows that when the arable land surrounding the Brook was ploughed in the late summer and early autumn of 1946, 1947 and 1948, micrococci were very numerous in all Zones (including the springs) in the autumn of 1946 and 1947 and dominated the flora in Zones I–III in the winter of 1947 (Zone IV was not sampled as workmen were clearing it out). Again, aerobic micrococci were present in all Zones (see Table 7) in the late summer of 1948, while microaerophilic cocci dominated the micro-flora in Zones II–IV in October 1948, the dominance persisting into December except in Zone IV.

Sheep were folded beside Zone III before ploughing there in the autumn of 1947 and 1948, and at the end of July and the beginning of August 1948 the land bordering the opposite bank to that on which sheep were folded was very heavily dressed with farmyard manure, far more heavily than at any other time during the investigation. In the middle of August 1948 there was a sudden and excessive growth of *Nasturtium officinale* (watercress) in Zone III which rapidly filled the reach (Pl. 1, fig. 5), spread far down into the upper third of Zone IV and by damming the water converted Zone II into a stagnant pool and drowned the springs. This excessive growth was attributed, in the absence of other evidence, to a conjunction of very heavy dressing of the land with manure with a fortnight of hot bright weather. The land was ploughed about the time the manure was applied, and as mentioned micrococci dominated the microflora in Zones II–IV in October 1948. The stagnation of the water caused by the damming of the current by watercress was possibly responsible however for the fact that the micrococci were universally microaerophilic, whereas at all other times they were aerobic. Although the watercress died back during the winter except for sporadic patches, aerobic spore-formers with some clostridia (i.e. *Cl. welchii*) were prominent in the microflora of Zones III and IV in the early spring of 1949 and may have been derived from the heavy dressing of manure.

It was previously mentioned that a large group of Gram-negative rods was isolated whose exact position was not determined. Lochhead, Timonin &

Table 7. *The dominant microflora of the Zones from October* 1946 *to October* 1948 *as shown by the routine sampling every* 2 *months*

(Incubation was at 22°. Dominance is assessed by the number of colonies showing the same characters as that of the organism whose morphology was shown by Gram-staining, viz. Mc = micrococci, G − = Gram-negative rods, G + = Gram-positive rods.)

		1946		1947						1948				
Zone	Month	Oct.	Dec.	Feb.	Apr.	June	Aug.	Oct.	Dec.	Feb.	Apr.	June	Aug.	Oct.
I	No. colonies on duplicate plates	10	30	1	2	4	4	28	80	1	17	121	4	1
	No. colonies stained by Gram's method	6	5	1	2	4	4	10	7	1	5	14	4	1
	Morphology	6 Mc	2 G− 2 Mc 1 G−	Mc	2 Mc	3 Mc 1 G−	3 G− 1 Mc	8 Mc 1 G− 1 G+	7 Mc	G+	4 Mc 1 G+	12 Mc 1 G− 1 G+	4 Mc	1 G+
	Dominance of colony types	Mc	.	.	Mc	Mc	G−	Mc	Mc	G+	Mc	Mc	Mc	
II	No. colonies on duplicate plates	22	8	19	6	6	29	44	400	4	2	17	16	29
	No. colonies strained by Gram's method	6	8	9	5	6	6	12	10	4	2	5	5	9
	Morphology	3 G− 2 Mc 1 G+	4 G− 1 G+ 3 Mc	9 G−	5 G−	5 G−	4 G+ 1 G− 1 Mc	8 G− 4 Mc	9 Mc 1 G−	2 Mc 2 G−	2 G−	4 G− 1 G+	4 Mc 1 G+	7 Mc 2 G−
	Dominance of colony types	Mc	.	G−	G−	G−	G+	G−	Mc	.	G−	G−	Mc	Mc
III	No. colonies on duplicate plates	26	10	25	6	32	9	40	430	6	25	48	726	25
	No. colonies stained by Gram's method	6	6	5	6	7	5	9	9	3	5	7	9	8
	Morphology	3 Mc 2 G− 1 G+	5 G− 1 Mc	5 G−	4 G− 1 Mc 1 G+	5 G− 2 Mc	3 G− 1 G+ 1 Mc	6 Mc 2 G− 1 G+	9 Mc	1 Mc 1 G− 1 G+	4 G− 1 G+	6 Mc 1 G−	8 Mc 1 G−	7 Mc 1 G−
	Dominance of colony types	Mc	Mc	G−	G−	G−	.	Mc	Mc	.	G−	Mc	Mc	Mc
IV	No. colonies on duplicate plates	262	10	14	386	36	100	120	*	11	18	33	682	9
	No. colonies stained by Gram's method	6	6	6	6	4	12	14	*	10	5	6	8	9
	Morphology	3 G+ 2 G− 1 Mc	4 G− 1 G+ 1 Mc	6 G−	4 G− 1 G+ 1 Mc	4 G−	11 Mc 1 G+	13 Mc 1 G−	*	7 Mc 2 G− 1 G+	4 G− 1 G+	5 G− 1 G+	5 Mc 3 G+	7 Mc 2 G−
	Dominance of colony types	.	G−	G−	G−	G−	Mc	Mc	*	Mc	G−	G−	Mc	Mc

* Not sampled—reach cleaned by workmen.

West (1940) showed that the rhizospheres of plants (notably cereal crops) are associated with a particular microflora; Gram-negative rods, for example, were found to predominate in the rhizospheres of oats, wheat and barley, and also in those of grass, e.g. clover. Gram-negative rods dominated the bacterial flora of Zone III when cereal crops were growing on its banks, and the same phenomenon was noted in Zones II and III in the spring and early summer of 1947 and 1948, and in Zone IV in the spring of 1948.

It is suggested that since soil constantly entered the Brook from climatic and other causes, the obscure group of Gram-negative rods isolated from the water possibly came from the rhizospheres of crop plants, which grew right up to the edge of the banks; moreover, the drainage of the subsoil was towards the stream. The dominance of Gram-negative rods in the bacterial flora as found in the routine sampling is shown in Table 7. The association between the bacterial flora of crop rhizospheres as given by Lochhead *et al.* (1940) and that found in the Brook from all examinations is given in Table 8.

Table 8. *Comparison of the dominant bacterial flora (as shown by all examinations of the current core), in the several Zones, with the supposed (*) microflora of the rhizospheres of the crops growing on either bank of the Brook*

			Crop rhizospheres microflora				Brook		
Zone	Year	Crop	G+ rods	G− rods	Micro-cocci	Season	G+ rods	G− rods	Micro-cocci
II†	1947	Barley	0	4	0	Spring	0	4	0
						Summer	0	4	0
	1948	Meadow grass	0	4	0	Spring	0	1	1
						Summer	0	4	0
III, upper third	1947	Wheat	3	4	1	Spring	0	4	0
		Barley	0	4	0	Summer	0	4	0
	1948	Meadow grass	0	4	0	Spring	0	4	0
		Oats	3	3	1	Summer	0	1	1
III, lower third	1947	Wheat	3	4	1	Spring	1	4	0
		Meadow grass	0	4	0	Summer	0	4	0
	1948	Linseed	0	4?	0	Spring	0	4	0
		Wheat	3	4	1	Summer	0	1	1
IV	1947	Beet	2	3	1	Spring	0	4	0
		Oats	3	3	1	Summer	0	0	1
	1948	Oats	3	3	1	Spring	0	4	0
		Wheat	3	4	1	Summer	0	0	1

* The composition quoted for the microflora is that given by Lochhead *et al.* (1940) for the crop in question.

† Only one bank of Zone 2 is given, as the other was covered by old permanent grassland throughout the investigation.

(Prevalence of the bacterial types is indicated roughly on the scale: 0 = absent, 1 = present, 2 = frequent, 3 = numerous, 4 = very numerous.

On this point, Dr A. G. Lochhead in a private communication observed that, regarding the extent to which changes are attributable to agricultural operations, from a quantitative standpoint the effect of soil is dependent upon such factors as size of stream, degree to which soil or soil washings are incorporated in the stream (rainfall, slope, etc.), and from a qualitative standpoint the chief effects would be due to change in cropping system, and heavy application of manure. In general, he concludes: 'I should be inclined to think that in the case of a stream the microbiology of the soil and that of fresh water are more nearly reflexions of each other than in the case of a lake or more quiescent body of water.'

If this possible association be confirmed, it is of great importance, for such Gram-negative bacterial rods are the chosen food selected by protozoa, especially by the bacteria-eating ciliate protozoa (Singh, 1941, 1946) and as found during this investigation, when an association between Gram-negative rods and bacteria-eating ciliates proved to be statistically significant. The number of protozoa would increase from the additional food supply, and that this would have a destructive effect upon pathogenic Gram-negative bacteria present is suggested by the following observations.

Pollution

No true *Esch. coli* were at any time isolated from the Brook. At the beginning of the inquiry the whole stream was examined for the presence of this organism by putting up dilutions in the appropriate media and reading the results by Macready's tables, positive samples being subsequently plated out on bile-salt agar. True faecal coliforms, however, were not isolated. Two further examinations for *Esch. coli* were made in the spring and autumn of 1948 at the mouth of the tributary which enters at the junction of Zones II and III (Fig. 1). These 'presumptive coliform' tests showed that the numbers of the organism were 2–25/100 ml. at the junction, but pollution could not be traced more than a few yards below the effluent mouth. This was considered remarkable since the tributary is formed by the union of two small streams which run through a farmyard and drain some old cottages rather more than half a mile from the Brook. The tributary is only half the width of the Brook but as long as the stretch of the latter investigated, with a very slow current that at times is negligible, and it contains farmyard and household waste.

Following the initial tests at the mouth of the tributary therefore, in the spring and late summer of 1948 a presumptive *Esch. coli* test was made along its length within 24 hr. of heavy rain, to detect how far pollution might be washed into the stream. Six points were sampled (Fig. 1), viz. on each tributary stream—points *A* and *B*; at *C*, approximately 100 yards below their union; at two equidistant points below this—*D* and *E*; and finally, at *F*, in the Brook approximately 50 yards below the mouth of the tributary. At the spring examinations the numbers of *Esch. coli* were (numbers/100 ml.) at points *A* and *B* 180+; at *C*, 90; at *D*–*F*, none. At the autumn test only points *C*–*F* were sampled, the numbers of presumptive coliforms/100 ml. being: 180+ at points *C* and *D*; 90+at *E*; 2–25 at *F* (Fig. 6). The writer was informed that during May and August 1938 a presumptive coliform test was carried out by the Public Health authorities in order to detect how far infection was carried into the Brook, but no degree of impurity was detected at either examination. It would appear therefore that in its course of about half a mile the tributary largely rids itself of coliform infection, and the agreement between the examinations carried out by the Public Health authorities in 1938 and the examinations in 1948 indicates that it has continued to do so.

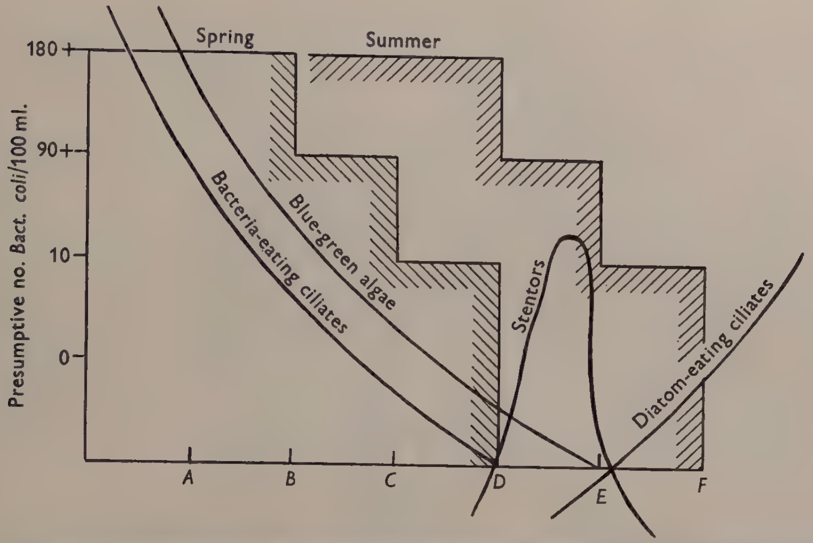

Fig. 6. The incidence of coliform infection in the tributary, tested at the six sampling points within 24 hr. of heavy rain, and the dominant ciliate Protozoa, in the spring and late summer of 1948.

As this fact was of considerable interest, a number of examinations were made to detect how it might occur. Turbulence followed by sedimentation was considered to be partly responsible. The Brook water of Zone II pours into Zone III at a right angle, cutting across the water entering from the tributary to impinge against the opposite bank of Zone III; the current from Zone II was so swift that the black mud of the tributary floor was commonly cut off as if with a knife. The turbulent flow from Zone II, it was thought, would dilute the bacterial numbers in the tributary water whilst sedimentation occurred at the opposing bank, additional sand and mud bars often being thrown up across the entrance to Zone III. The effect was shown by sampling the bacterial numbers in Zone II, at points across the junction of Zone II, the tributary and Zone III, and at 50–100 yards downstream in Zone III (Fig. 7). But these physical factors were regarded as subordinate to the activity of protozoa.

The phagocytic activity of ciliate protozoa in particular was regarded as the major factor in clearing pollution. It has been mentioned that in the Brook an association between Gram-negative rods and bacteria-eating ciliate protozoa proved to be statistically significant. It was further noted here that whereas bacteria-eating ciliates such as *Colpoda* and *Colpidium* were associated with strains of *Pseudomonas* and multiplied when they were abundant, Paramoecidae were associated with typical strains of *Esch. coli*. Taylor & Strickland (1938) used a strain of *Pseudomonas* as food for their cultures of *Colpoda duodenarii*. In the tributary the curve of coliform infection was closely followed by the curve of bacteria-eating ciliate protozoa, notably *Paramoecium caudatum*,

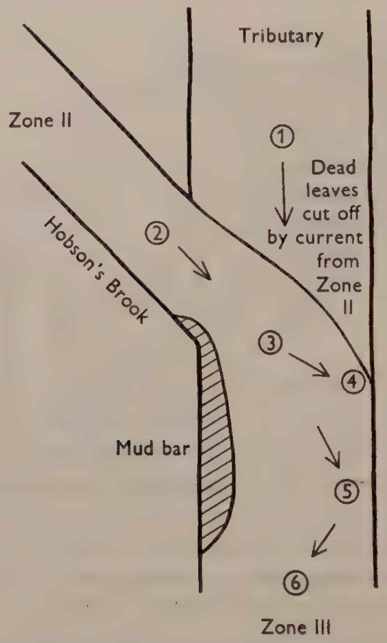

Fig. 7. The viable counts at 22° at the junctions of Zone II and III and the tributary in May 1948. Diagram of the junction and showing observation points referred to in the table below. Distance from point 2 to point 6 approximately 5 yd. Arrows indicate direction of flow.

Point	Temp. (° C.)	pH	O^2 (ml./l.)	Viable count (22°) ($\times 10^3$/ml.)
1	15	7·3	4·1	10
2	12	7·3	6·9	7
3	13	7·3	4·8	9
4	15	7·3	—	11
5	15	7·3	—	19
6	15	7·3	—	14

At point 1 diatoms and diatom-eating ciliate protozoa were found. No infusoria at points 2–5. At point 6 bacteria-eating ciliate protozoa appeared.

P. bursaria and other forms of Paramoecidae. These organisms were present almost to the exclusion of all others wherever pollution was highest (Fig. 6). Indeed, the typical microscopical picture of the tributary water, its heavy organic-matter content, faecal pollution and teeming population of Paramoecidae was very reminiscent of that found in 1844 in the Thames near sewer mouths by Hassall (1850, 1855), whose observations prompted this inquiry. Further, whenever surface water from prolonged heavy rain or heavy storms flooded down the tributary and turbidity was seen to enter the Brook, populations of bacteria-eating ciliate protozoa, especially of Paramoecidae, appeared in the upper third of Zone III and subsequently disappeared.

Effects of the biota of the Brook

In the Brook there was at all times an abundant phytoplankton of diatoms. There were also 'blooms' of blue-green Algae (*Oscillatoria*) which floated down the stream. In addition there were the flowering plants (*Phanerophyta*) characteristic of alkaline streams, e.g. *Nasturtium officinale*, *Veronica anagallis*, *Apium nodiflorum* and *Ranunculus trichophilus*. These were seasonal in appearance, being most abundant in autumn in Zone III. Bacterial numbers were always greatest in relation to plants, and highest of all in relation to blue-green algae, 'blooms' of which floated down from Zone II to Zone IV in October 1946, March 1947, and January 1948 (Table 9). These high numbers did not appear to be reflected in the routine samplings, since it was usually found that they did not persist more than a foot or two downstream below the plant, or on occasion immediately in front of it upstream. The same was generally true of all fixed objects in the current core (Table 9). Nevertheless, care was taken wherever possible to sample from clear water not less than 4–6 ft. from any obstacle. There was some indication that chromogenic bacteria (other than strains of *Chromobacterium violaceum* and *Flavobacterium* spp.) were more common near plants and other obstacles than elsewhere (Table 9).

There was no free-floating zooplankton in the stream. Ciliate protozoa were most abundant on objects in the centre of the stream, for example on the leaves of plants, but their numbers were depressed each year by a late summer peak of dipterous larvae, e.g. Simulidae (blackfly) and Chironomidae (midges), which occupied the centre of the stream. Barker (1946) found an identical relation in sewage filter-beds, where seasonal peaks of fly output were accompanied by a decrease of ciliate protozoa. This decrease in numbers of ciliates would be expected to raise the numbers of those bacteria on which they feed, and this may have contributed to obscure any diminution in numbers caused by higher summer temperatures.

The close association found during the investigation between the bacteria of the stream and the bacteria of the arable land through which it runs, the relation of bacterial numbers and types to plants seasonal in appearance, and the annual decrease caused by fly larvae of ciliate protozoa which feed upon bacteria, emphasize the importance of studying the bacteria of such waters in relation to their ecology as a whole.

The writer is glad to express his gratitude to the Department of Scientific and Industrial Research for a grant which enabled the study to be carried out, and to Prof. F. T. Brooks, F.R.S., for permission to work at the School of Botany.

Table 9. *Viable counts at 22° and chromogenic bacteria in the vicinity of fixed objects in the stream*

			Number of bacteria (10^3/ml.)				Chromogenic bacteria	
Date	Zone	Object	At object	3 ft. up-stream	3 ft. down-stream	Current core	At object	Current core
31. i. 47	III	Edge of brick arch	5	8	4	4	.	.
28. i. 47	III	Rotting sack	36	4·5	9·5	.	.	.
5. ii. 47	III	Rotting sack	72	141	10	.	14	5
10. ii. 47	II	Mass of rotting vegetation	44	19	136	.	7	5
17. x. 46	IV	Blue-green alga	500	262	.	.	.	.
29. iv. 47	II	Grass-green algae on sandbar	688	52	34	.	13	0
29. vii. 47	III	Grass-green alga	142	11	.	15	20	.
9. x. 47	III	Alga (*Spirogyra*)	736	40	.	16	.	.
9. x. 47	IV	Alga (*Cladophora*)	520	1200	.	.	.	.
31. x. 47	III	Diatoms	468	40	.	.	.	.
31. x. 47	IV	Diatoms	168	120	.	.	.	.
9. ii. 48	II	Old grass green algae	40	12·5	11	3	6	.
11. iii. 47	IV	Flowering plant	68	4	.	.	16	.
10. v. 47	III	Flowering plant	56	11	54	9	12	.
5. vi. 47	II	Flowering plant	44	.	.	.	1	.
5. vi. 47	III	Flowering plant	16	.	.	.	.	.
5. vi. 47	IV	Flowering plant	52	.	.	.	12	.
10. vi. 47	II	Flowering plant	88	.	.	6 at the bi-monthly test 13/6	.	.
10. vi. 47	III	Flowering plant	78	.	.	32 at test above	4	3
10. vi. 47	IV	Flowering plant	148	.	.	36 at test above	6	.
5. vii. 48	III	Flowering plant	87	.	.	11	4	.

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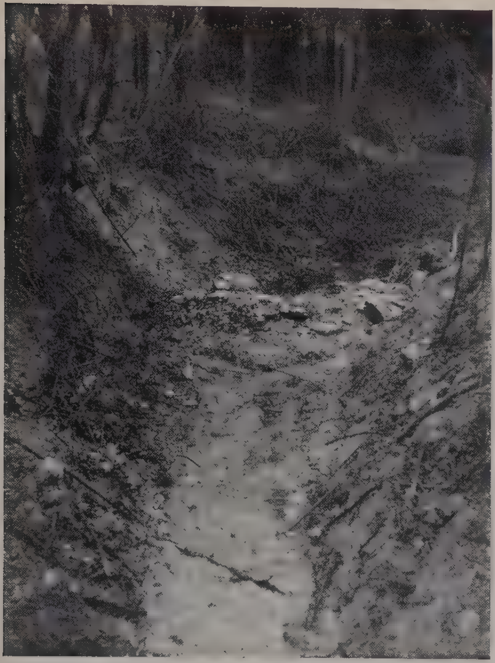
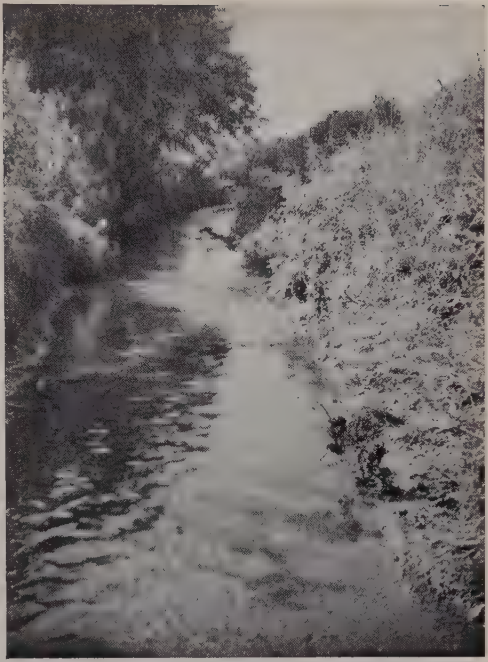


Fig. 1



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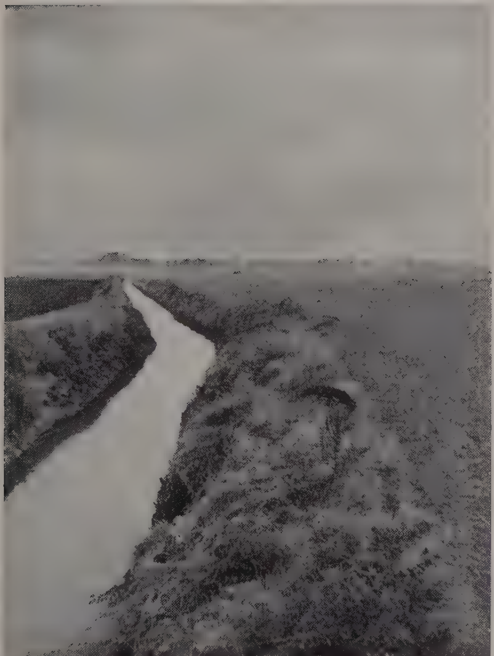

Fig. 3

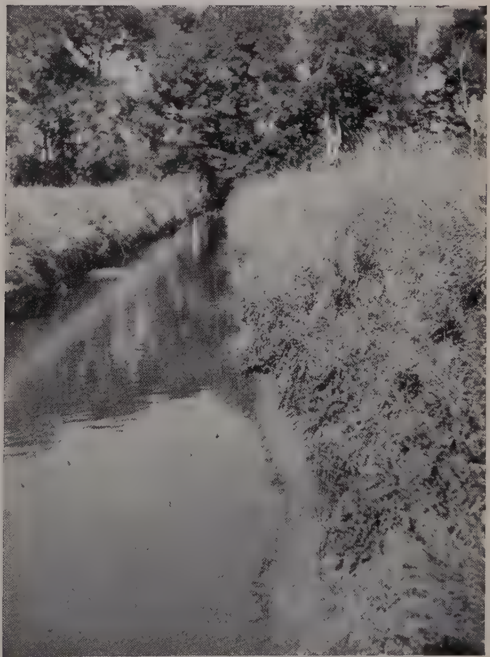

Fig. 4

E. Gray—Bacterial ecology of a chalk stream. Plate 1

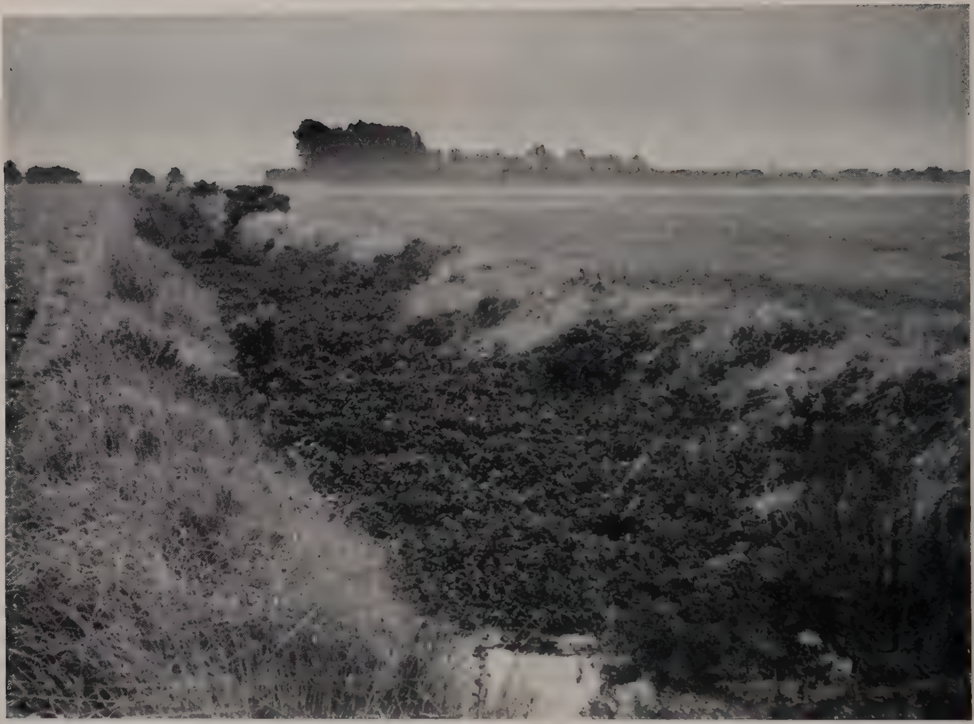

Fig. 5

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. The Nine Wells springs. The particular spring sampled during the investigation is shown. The water breaks through fissures in a chalk face (hidden by the right hand bank) with sufficient force to produce a spurt of foam clearly visible near the right centre of the photograph.

Fig. 2. The gravel reach of Hobson's Brook. (Zone II).

Fig. 3. The centre of the rural reach (Zone III) in winter 1947.

Fig. 4. Zone IV in summer 1948, looking towards the main road (in the trees behind) which limited the rural reach of the Brook. At the end of the reach is the graduated post for measuring the water volume.

PLATE 2

Fig. 5. The abnormal growth of *Nasturtium officinale* in the centre of the rural reach (Zone III) following heavy manuring of the arable land on the banks.

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Non-competitive Sulphanilamide Antagonists for Yeasts

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SUMMARY: Sulphanilamide inhibition of the growth of certain yeasts and yeast-like organisms was overcome in a non-competitive manner by methionine, adenine and histidine, which are therefore regarded as end-products of enzyme systems for which *p*-aminobenzoic acid is essential. The methionine system was the most sensitive to sulphanilamide, followed in order by those of adenine and histidine.

In the presence of methionine, adenine and histidine together, the test organisms were still sensitive to sulphanilamide, implying that *p*-aminobenzoic acid is concerned in still another system, or systems. The nature of these systems is obscure, but the inhibition is not overcome by purines, pyrimidines or pteroylglutamic acid, nor by single amino-acids, except in one case when DL-cysteine showed some effect. In another case, a low concentration of choline chloride was effective.

Evidence of the vital functions of *p*-aminobenzoic acid (*p*-AB) may be obtained by the method of inhibition analysis using sulphanilamide (S-amide) (Shive & Roberts, 1946). The biosynthesis of methionine and of the purine skeleton seems to be dependent on enzyme systems in which *p*-AB plays an essential role, since methionine and purines overcome S-amide inhibition in a non-competitive manner (Harris & Kohn, 1941; Snell & Mitchell, 1942; Lampen & Jones, 1947). This conclusion is supported by other studies which showed that *p*-AB can be replaced by purines and amino-acids in the growth of mutant strains of *Escherichia coli* (Lampen, Jones & Roepke, 1949) and by adenine, methionine and histidine for the growth of a yeast (Cutts & Rainbow, 1950).

In other cases, S-amide inhibition is overcome by relatively high concentrations of thymine or by relatively low concentrations of pteroylglutamic acid (PGA). It is therefore concluded that PGA is concerned in the biosynthesis of thymine (Lampen & Jones, 1947; Winkler & de Haan, 1948). As a result of extensive inhibition analysis of *Esch. coli*, the latter workers found that serine is an S-amide antagonist, and Lampen *et al.* (1949) showed that other amino-acids have some effect. In the present study, purines, pyrimidines, amino-acids, PGA and choline were tested as antagonists of S-amide bacteriostasis of some yeast and yeast-like organisms, and the results compared with those already obtained by a different technique (Cutts & Rainbow, 1950).

METHODS

The basal medium used was that described by Cutts & Rainbow (1950).

Test organisms and inocula. Four test organisms were used; cultures of each were maintained on malt agar slants and transferred 2–3 times weekly as required.

(*a*) The strain of *Saccharomyces cerevisiae*, designated 'Yeast 47' by Rainbow (1948). This organism requires exogenous sources of biotin, pantothenate and *p*-AB. Inocula were prepared by transferring a little surface growth from a malt agar slant to 6 ml. of basal medium containing 0·1 μg. *p*-AB/ml. and incubating at 25° for 18–24 hr. The cells were then washed twice (centrifuge) in 6 ml. portions of sterile 0·85 % saline and finally suspended in a further 6 ml. portion.

(*b*) A strain of top fermentation brewer's yeast (*S. cerevisiae*) isolated by us from a brewery pitching yeast and designated 'Yeast 70'. This strain required biotin and inositol, synthesized pantothenate slowly, but was independent of exogenous *p*-AB. Inocula were suspensions in sterile saline of a little of the surface growth from a malt agar slant.

(*c*) *Kloeckera brevis*, B 768 (Emery, McLeod & Robinson, 1946). This strain was independent of exogenous *p*-AB. Inocula were prepared as for (*b*) above.

(*d*) *Schizosaccharomyces octosporus*, an organism requiring for growth exogenous adenine, methionine and histidine, but not *p*-AB (Northam & Norris, 1951). Inocula were grown in basal medium supplemented with (μg./ml.): DL-methionine (100), adenine HCl (50) and L-histidine HCl (25) and otherwise treated as in (*a*) above.

Auxanographic tests were carried out as previously described (Cutts & Rainbow, 1950). For each series, the following substances were tested for ability to overcome sulphanilamide inhibition: *purines and pyrimidines*—adenine, guanine, xanthine, cytosine, thymine and uracil; *amino-acids*—DL-alanine, L-arginine, L-asparagine, L-aspartic acid, DL-cysteine, L-cystine, glycine, L-glutamic acid, L-histidine, L-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-norleucine, DL-norvaline, L-proline, DL-phenylalanine, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine and DL-valine; other substances—*p*-AB, PGA and choline chloride.

Tests in liquid media were carried out as described by Northam & Norris (1951). Observations were made on duplicate cultures.

RESULTS

Inhibition of Yeast 47

Auxanographic tests were carried out to identify substances which could overcome the inhibition of the growth of Yeast 47 by sulphanilamide (S-amide). The substances listed above were first tested in the basal medium containing 1000 μg. S-amide and 0·5 μg. *p*-AB/ml. In this medium, little growth took place after 48 hr. incubation, during which period observations were made. After 96 hr. growth was heavy and general on all plates, except around the point of application of norleucine. The results are recorded in Table 1. Apart from *p*-AB, DL-methionine, adenine, guanine and DL-cysteine each overcame S-amide inhibition, whilst norleucine accentuated the inhibition.

For the second series of auxanographs, DL-methionine adenine and guanine were added singly to portions of basal medium containing a concentration of *p*-AB decreased to permit inhibition of growth by 1000 μg. S-amide/ml. In these tests, S-amide inhibition was overcome by adenine or by guanine in the medium containing methionine, or by DL-methionine in the media containing adenine or guanine. Norleucine was again inhibitory in the two latter media.

In basal medium containing S-amide, *p*-AB, DL-methionine and adenine, inhibition of Yeast 47 was overcome only by L-histidine and by *p*-AB itself. By including L-histidine HCl in the basal medium, as well as adenine and methionine, and diminishing the *p*-AB concentration still further to permit of inhibition, Yeast 47 was found too tolerant of S-amide for further results to be obtained, unless the inoculum used for the tests was one of deficient cells obtained by growth in the defined *p*-AB-free medium described in (*d*) above. In this way, DL-cysteine was shown to overcome S-amide inhibition in the presence of methionine, adenine and histidine.

For the final auxanographs, adenine, methionine, histidine and DL-cysteine were included in the basal medium containing S-amide and *p*-AB. Under these conditions and using an inoculum from the *p*-AB free defined medium,

Table 1. *Antagonists of sulphanilamide inhibition of various organisms*

(Results were obtained by auxanograph technique; observations up to 48 hr. of incubation at 25°.)

Basal medium containing			
Supplements (μg./ml.)	S-amide (μg./ml.)	*p*-AB (μg./ml.)	S-amide inhibition overcome by
	Yeast 47		
None	1000	0·5	Adenine, guanine, DL- methionine, DL-cysteine, *p*AB
DL-Methionine (100)	1000	0·1	Adenine, guanine, *p*-AB
Adenine HCl (50)	1000	0·1	DL-Methionine, *p*-AB
Guanine (50)	1000	0·1	DL-Methionine, *p*-AB
DL-Methionine (100) adenine HCl (50)	1000	0·005	L-Histidine HCl, *p*-AB
DL-Methionine (100)* adenine HCl (50)* L-histidine HCl (25)*	1000	0·001	DL-Cysteine HCl, *p*-AB
DL-Methionine (100)* adenine HCl (50)* L-histidine HCl (25)* DL-cysteine HCl (250)*	1000	0·0003	*p*-AB
	Yeast 70		
None	1000	0	Adenine, guanine, DL-methionine, *p*-AB
DL-Methionine (100) adenine HCl (50)	3000	0	L-Histidine, L-leucine, L-tyrosine, *p*-AB
DL-Methionine (100) adenine HCl (50) L-histidine HCl (25)	3000	0	No demonstrable inhibition
	Kloeckera brevis		
None	1000	0	DL-Methionine, PGA, *p*-AB
DL-Methionine (100)	1000	0	Adenine, *p*-AB
Adenine HCl (50)	3000	0	No demonstrable inhibition
	Schizosaccharomyces octosporus		
DL-Methionine (100)* adenine HCl (50)* L-histidine HCl (25)*	1000	0	Choline chloride, *p*-AB

* Tests carried out using an inoculum grown on a *p*-AB-free medium.

Yeast 47 was still sensitive to S-amide, the inhibition being overcome only by *p*-AB of all the substances tested.

Non-competitive nature of the anti-S-amide action of purines and amino-acids. For Yeast 47, a competitive relation was found to exist between *p*-AB and concentrations of S-amide between 300 and 3000 μg./ml. For lower concentrations of S-amide, the ratio of the concentrations (S-amide)/(*p*-AB) required just to restore full growth (i.e. the antibacterial index) diminished, as might be expected when the enzyme systems involved are no longer saturated with respect to *p*-AB and S-amide. At the other extreme, the inhibition due to 10,000 μg. S-amide/ml. was not completely overcome by *p*-AB.

The non-competitive nature of the action of methionine, adenine and histidine in overcoming the S-amide inhibition of Yeast 47 was demonstrated in liquid medium containing 1000 μg. S-amide/ml. Fig. 1 shows that methionine behaved as a non-competitive antagonist. The concentration of *p*-AB required to restore full growth was decreased from 1·0 to 0·3 μg. *p*-AB/ml. (approximately a threefold increase in the antibacterial index) by 100 μg. of DL-methionine/ml. This concentration of methionine partially overcame the inhibition in the presence of 0·1 μg. *p*-AB/ml., but higher methionine concentrations, which would be expected to be more effective if the relation were competitive, were no more effective.

The non-competitive anti-S-amide activities of adenine and histidine are shown in Figs. 2 and 3 respectively. The effect of adenine was evident in a medium containing 100 μg. DL-methionine/ml., 30 μg. adenine HCl/ml. producing a further threefold increase in the antibacterial index. In a separate experiment, guanine was found to be equally effective. To demonstrate the non-competitive anti-S-amide activity of histidine, the presence in the medium of both DL-methionine (100 μg./ml.) and adenine HCl (100 μg./ml.) was necessary. In this case, the inhibition in *p*-AB-free medium was partially overcome by 100 μg. L-histidine HCl/ml.; 1000 μg. L-histidine/ml. was no more effective. Thus for Yeast 47 methionine, adenine and histidine are non-competitive S-amide antagonists, which suggests that these are end-products of enzymic systems for which *p*-AB is essential.

The order in which methionine, adenine and histidine exert antisulphonamide activity. The effects on the antibacterial index of additions of methionine, adenine and histidine, singly and in all possible combinations, to the basal medium was determined by tube tests (Table 2). The antibacterial index was almost unaltered by additions of adenine or histidine, or by adenine + histidine, but was increased some 2·5 times by methionine. The effect of histidine and methionine together was no greater than that of methionine alone, but the combination adenine + methione resulted in a further 2·5-fold increase of the index. The effect of histidine was evident only when adenine and methionine are both present.

It therefore appears that the anti-S-amide actions of these substances are exerted in a definite order, the methionine system being the most sensitive to S-amide, that of adenine intermediate and that of histidine the least sensitive. The successive effects of the three substances is shown in Fig. 4.

Inhibitions of other yeasts

To examine whether the results obtained with Yeast 47 were of more general significance, other yeasts and yeast-like organisms were tested. Only qualitative tests were made, by means of the auxanographic technique, with the minor

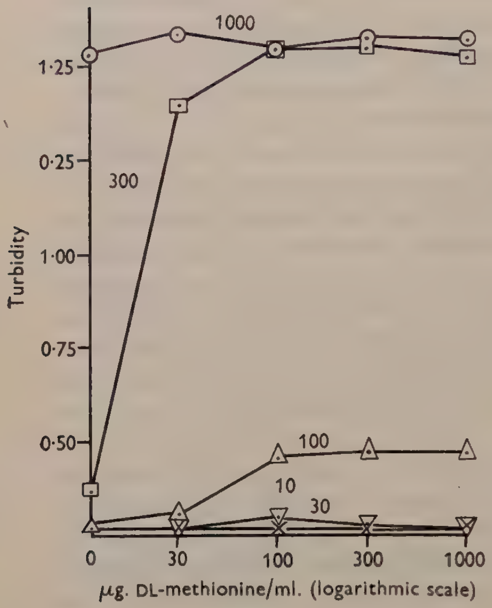

Fig. 1

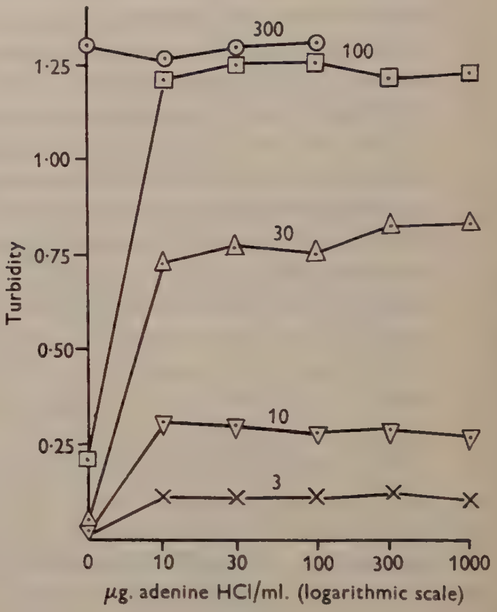

Fig. 2

Fig. 1. S-amide inhibition (1000 μg. S-amide/ml.) of Yeast 47 overcome by DL-methionine. The numbers above the curves show the respective concentrations of *p*-AB (mμg./ml.).

Fig. 2. Inhibition of Yeast 47 in presence of 1000 μg. S-amide + 100 μg. DL-methionine overcome by adenine at various concentrations of *p*-AB (*p*-AB in mμg./ml. as shown by the numbers above the respective curves).

Table 2. *Effects of methionine, adenine and histidine on the antibacterial index with Yeast 47*

(Antibacterial index = 1000/μg. *p*-AB/ml. required just to restore full growth in 48 hr. at 25°. Basal medium contained 1000 μg. S-amide/ml.)

Supplements (μg./ml.)	Antibacterial index (10^3)
Nil	1·0
Adenine HCl (50)	1·0
L-Histidine HCl (25)	1·0
Adenine HCl (5) + L-histidine HCl (25)	1·0
DL-Methionine (100)	2·5
DL-Methionine (100) + L-histidine HCl (25)	2·5
DL-Methionine (100) + adenine HCl (50)	6·0
DL-Methionine (100) + adenine HCl (50) + L-histidine HCl (25)	60·0

modification that, since none of the organisms tested required a source of exogenous p-AB, this substance was omitted from the basal medium, in order that the sensitivity to S-amide should not be diminished. The results are recorded in Table 1.

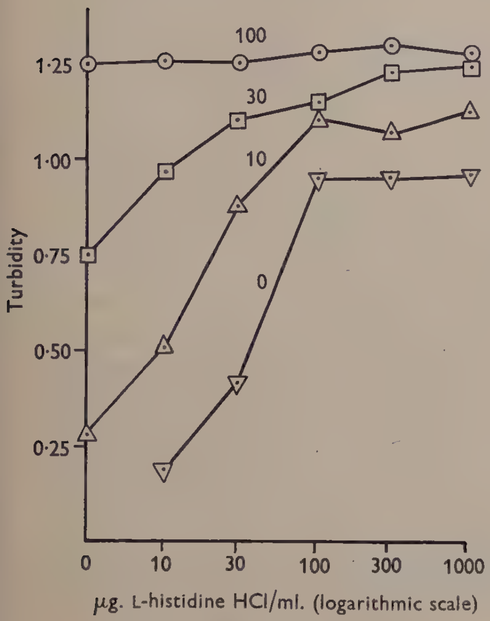

Fig. 3

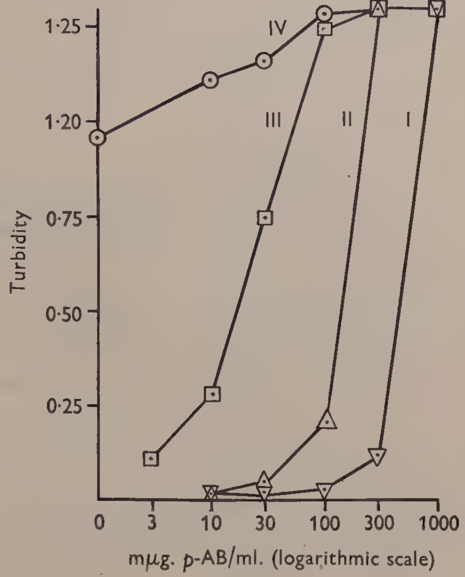

Fig. 4

Fig. 3. Inhibition of Yeast 47 in presence of S-amide (1000μg./ml.) + DL-methionine (100μg./ml.) + adenine HCl (100μg./ml.) overcome by L-histidine at various concentrations of p-AB. p-AB concentrations are indicated by the numbers (mμg./ml.) above the respective curves.

Fig. 4. Inhibition of growth of Yeast 47 in basal medium + 1000μg. S-amide/ml. overcome by p-AB under various conditions. Curve I, no additions; curve II, +100μg. DL-methionine/ml.; curve III, +100μg. each of DL-methionine and adenine HCl/ml.; curve IV, +100μg. each of DL-methionine, adenine HCl and L-histidine HCl/ml.

S-amide inhibition of Yeast 70 resembled that of Yeast 47 with respect to methionine, adenine and histidine, except that inhibition in the presence of methionine and adenine was overcome by L-leucine and L-tyrosine, as well as by L-histidine. Cutts & Rainbow (1950) showed that the p-AB-sparing effect of suboptimal concentrations of methionine was much enhanced by leucine and tyrosine, and in the present case these amino-acids may act by virtue of their methionine-sparing effect. This conclusion is supported by the fact that, on raising the concentration of DL-methionine to 250μg./ml. and repeating the test with Yeast 70, no anti-S-amide activity by L-leucine or L-tyrosine could be detected.

With *Kloeckera brevis*, S-amide inhibition was overcome by methionine and adenine; but in the presence of both these substances, the organism was too tolerant of S-amide to permit of further inhibition analysis. The action of PGA as an anti-S-amide substance was approximately competitive over a limited range, a relatively high concentration (3 μg./ml.) being required to overcome the inhibition by 1000 μg. S-amide/ml. This suggested that the activity of the PGA was due to its conversion by *K. brevis* into free *p*-AB (cf. Koft, Sevag & Steers, 1950).

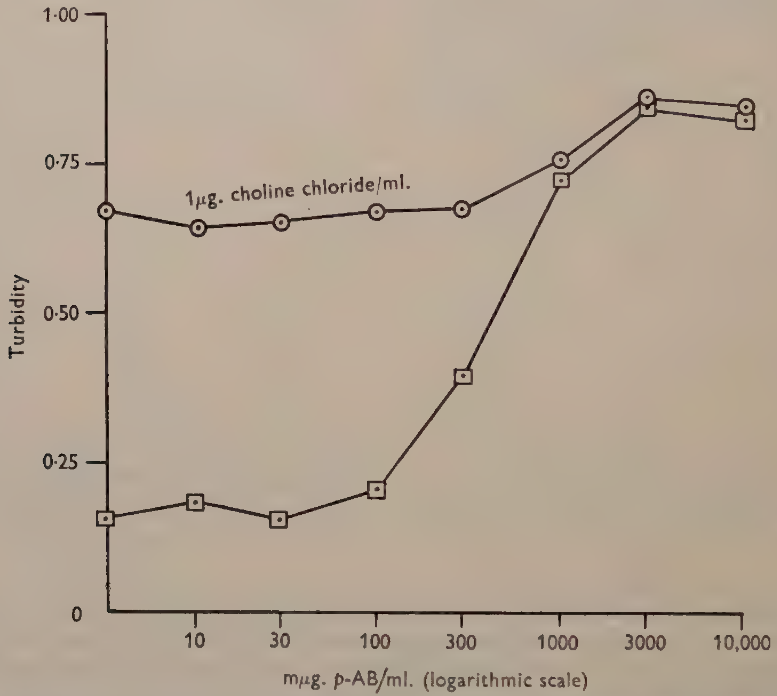

Fig. 5. Inhibition of growth of *Schizosaccharomyces octosporus* in basal medium + 1000 μg. S-amide/ml. + 100 μg. DL-methionine/ml. + 50 μg. adenine HCl/ml. + 25 μg. L-histidine HCl/ml. Inhibition overcome by *p*-AB in the presence and absence of 1 μg. choline chloride/ml.

Schizosaccharomyces octosporus did not require exogenous *p*-AB for growth, but had to be provided with adenine, methionine and histidine, the syntheses of which compounds could not therefore be blocked by S-amide. Nevertheless, the organism showed inhibition by S-amide, which was overcome by 1 μg. choline chloride/ml. (Fig. 5), although the growth rate was then subnormal; concentrations of choline chloride exceeding 1 μg./ml. had no greater effect.

DISCUSSION

As non-competitive antagonists of S-amide inhibition, methionine, adenine and histidine may be regarded as end-products of the activity of certain enzyme systems for which *p*-AB is an essential component. The results confirm those of Cutts & Rainbow (1950), who found that relatively high concentrations of methionine, adenine and histidine could replace *p*-AB (albeit not completely) for the growth of Yeast 47. With Yeast 47, the difference in sensitivity to S-amide between the systems involved in the syntheses of methionine, adenine and histidine suggests that three different enzyme systems, each requiring *p*-AB, are involved. That *p*-AB is concerned in the synthesis of an intermediate, common to all three substances, seems less likely.

The inhibitory effect of norleucine in the auxanographs of Yeasts 47 and 70 on media containing no methionine is ascribable to the synergistic effect exercised by norleucine and suphonamides and which may be due to interference by norleucine with methionine metabolism, since the latter two substances are structurally analogous.

Guanine is as effective as adenine in overcoming inhibition by S-amide with Yeasts 47 and 70, but it cannot replace adenine in *p*-AB-free medium for Yeast 47 (Cutts & Rainbow, 1950). Thus, Yeast 47 can convert adenine to guanine, but not guanine to adenine. Guanine may therefore exert its action as an antisulphonamide by 'sparing' the product of a reaction inhibited by S-amide, viz. adenine. Comparison may be made with the work of Kerr, Seraidarian & Brown (1951) who found that, whereas isotopically labelled adenine was incorporated into the ribonucleic acid of *Torulopsis utilis* both as adenine and guanine, labelled guanine appeared only as guanine.

There is no evidence that PGA is an essential cell constituent for the yeasts studied. Other tests with Yeast 47, not reported here, confirm that PGA will neither replace *p*-AB nor raise the level of growth obtainable in the presence of methionine, adenine and histidine in *p*-AB-free medium. The explanation of the inactivity of synthetic PGA for yeasts possibly lies in Woods's (1948) suggestion that synthetic PGA may not be identical with (or convertible to) all natural forms of 'folic acid'.

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On *Ruminococcus flavefaciens*, a Cellulose-decomposing Bacterium from the Rumen of Sheep and Cattle

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SUMMARY: Two strains of *Ruminococcus flavefaciens*, an important cellulose-decomposing bacterium, were isolated, one from the rumen of a sheep, the other from the rumen of a cow. Pure cultures were obtained by using the dilution method in agar media containing a strip of filter-paper. These strictly anaerobic, Gram-positive streptococci attack cellulose and cellobiose, but not starch, maltose, lactose or xylose. Only one strain could use glucose. Colonies on cellulose media were characterized by the formation of a yellow pigment; in cellobiose media the colonies were white. Growth on cellulose was favoured by addition of *Clostridium sporogenes* or a certain amount of sterilized medium in which *Cl. sporogenes* had previously grown.

Estimations of the end-products of fermentation of cellulose and cellobiose showed that at least 25 % of the carbon could be recovered as succinic acid, *c.* 23 % as acetic acid and *c.* 10 % as formic acid; ethanol was absent and gas formation very limited. A description of the genus and the species is given.

Our knowledge of the bacteria that effect the breakdown of cellulose in the rumen of ruminants is still very meagre. Hungate (1947) was the first to obtain pure cultures of several types of these bacteria, in the form of asporogenous rods or cocci. Using a similar technique the present author (1948) isolated from a bovine rumen a cellulose-decomposing streptococcus, to which the name *Ruminococcus flavefaciens* was given. This is a small Gram-positive, obligate anaerobic streptococcus which forms yellow colonies on filter-paper. This organism must be considered one of the predominant species of cellulose-decomposing bacteria in the rumen. As growth in pure culture was very poor, little information was then obtained about them and an analysis of the products of their metabolism was impossible. In combined culture with *Cl. sporogenes*, however, cellulose was rapidly attacked by the streptococci, acetic and succinic acids being the main end-products. The present work was carried out with two other strains of *Rum. flavefaciens*, and more information obtained about the species.

During this investigation a further paper was published by Hungate (1950), in which he described more closely four bacterial types that play an important role in the decomposition of cellulose in bovine rumen. Two of these are cocci; it is not excluded that one of them is identical with *Rum. flavefaciens*.

MATERIAL AND METHODS

Strains. The present work was carried out with two strains of *Rum. flavefaciens*: strain S from the rumen of a fistulated sheep; strain D from the rumen of a slaughtered cow. Strain S was investigated at the Department of

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Bacteriology of Sheffield University, and strain D at the Laboratorium voor Microbiologie of the Technical University at Delft, Holland.

Cultivation. For cultivation and purification the dilution method in agar media (Sijpesteijn, 1948) was used in somewhat simplified form.

Conical flasks (100 ml.) contained 25 ml. of a solution of the following composition (in %, w/v): Davis's New Zealand agar (1·5), K_2HPO_4 (0·06), KH_2PO_4 (0·04), $(NH_4)_2SO_4$ (0·04), NaCl (0·12), $MgSO_4.7H_2O$ (0·02), $CaCl_2.6H_2O$ (0·02). To each flask 19 ml. tap water was added. The medium was boiled out and a reducing agent added, either 25 mg. Na thiolacetate per flask in the case of strain S, or 50 mg. L-cysteine HCl adjusted to pH 7, for strain D. After autoclaving, 3 ml. yeast autolysate and 3·5 ml. 7 % $NaHCO_3$ were added per flask to give a final concentration of 6 % (v/v) and 0·5 % (w/v) respectively in the final liquid volume of *c.* 50 ml. The bicarbonate solution was sterilized by filtration. The filled flasks were put in a water-bath at 38–40° and gassed for about 5 min. with CO_2 freed from traces of oxygen by passage over reduced copper turnings heated to *c.* 400°. The medium was subsequently distributed between six sterile tubes each containing a strip of Whatman no. 1 filter-paper. The medium remained liquid at the temperature of the water-bath. The New Zealand agar proved to be very useful in this procedure because it gelled at a lower temperature than Japanese agar at the same concentration. One of the tubes was then inoculated with 0·05 ml. rumen contents which had been previously filtered through muslin. The tube contents were mixed thoroughly by passing oxygen-free CO_2 through the medium by means of a Pasteur pipette inserted between the plug and the wall of the tube, with the tip of the pipette at the bottom of the tube. Another tube was then inoculated with 0·5 ml. of the first tube, and, after mixing, 0·5 ml. was transferred to a third tube, and so on. After inoculation each tube was gassed with CO_2 for about 4 min. This proved sufficient to ensure saturation of the medium with CO_2 (producing a final pH *c.* 6·6), to remove traces of oxygen from the medium, and to replace the air above it by CO_2. Methylene blue became decolorized almost immediately when added to this medium. During the final gassing procedure the plug was pushed into the tube, the Pasteur pipette withdrawn, and a rubber stopper quickly fitted to the tube. The tubes were then put into cold water to solidify the agar and after sealing the stoppers with paraffin-wax, incubated at 38°. This method is similar to that used by Hungate (1947, 1950), who, however, appears to take still more rigid precautions to prevent access of oxygen to his cellulose-decomposing bacteria. The method described above was adequate for the organisms we were dealing with.

Purification. The bacteria were purified as follows. Some days after inoculation with rumen contents colourless and yellow colonies which invariably consisted of cocci became visible on the paper strips. Yellow colonies of cellulose-decomposing rods such as Hungate (1950) described were never encountered. A yellow colony from the tube which had received the most dilute inoculum was used for each transfer. The bottom was cut from the tube and the agar column pushed out into a sterile Petri dish. The attacked strip of paper was then lifted from the agar with a pair of forceps and placed in another

Petri dish on a layer of agar. As the organisms do not destroy the paper completely a yellow colony could then easily be picked out with a needle and transferred into a tube of fresh medium.

Strain S was subcultured from one of three yellow colonies obtained by inoculation of a tube with 0·003 ml. rumen liquid. It was pure after six serial transfers. Microscopically, contaminants were noticeable neither in the agar nor in the yellow colonies. Moreover, pieces of the agar or the filter-paper did not give rise to any growth after anaerobic incubation in peptone broth or meat medium.

Strain D was obtained from a tube that had been inoculated with 0·00045 ml. rumen liquid. On the paper strip three yellow colonies and one colourless colony developed. A pure culture was obtained after eight transfers from one of the yellow colonies. Since growth became very scanty as the culture was purified, the medium was modified as described below. The purity of the culture was checked microscopically as well as by incubation in peptone broth.

CULTURAL CHARACTERISTICS AND CULTIVATION

Growth on cellulose

Morphologically, strains S and D resembled closely the previously described (Sijpesteijn, 1948) strain G of *Rum. flavefaciens*. They were Gram-positive, catalase-negative streptococci with chains of 2, 4 or 8 cells; cell diameter 0·8–0·9 μ (Pl. 1, fig. 1). In strain D chain formation was less pronounced than in the other strains and single cocci were observed. Capsules were not noticed in strain S, but were frequent in strain D. Growth on cellulose was accompanied by pale yellow pigmentation of the paper strip, which was never so completely digested that holes were formed.

The growth of both these strains was better than that of strain G had been, although the filter-paper and the agar used at present are not superior for strain G to those previously used. Growth of the purified strains S and D was not so good as in the initial subcultures when many contaminants were still present. We return to this problem below.

Growth occurred in agar media with filter paper or finely divided cellulose, prepared according to Scales (1916), Fuller & Norman (1942), or Hungate (1947). Cotton-wool and cellophan were also attacked.

About 10 days after inoculation the cultures showed a remarkable phenomenon. Tiny white colonies became visible in the agar adjacent to the paper strip. Their diameter never exceeded *c.* 0·3 mm. and they grew within some 5 mm. of a colony on filter paper. Their size was larger the nearer they were to the paper. Microscopically, they only differed from the cellulose bacteria by their longer chains. The occurrence of minute secondary colonies was described for strain G (Sijpesteijn, 1948), and it was then shown that transfer of such a colony to the usual cellulose medium gives rise once more to many yellow colonies on the paper. Obviously the colonies consisted of cellulose-decomposing bacteria, and we assume that they grew on account of a diffusible substance produced from cellulose by the parent colony. Part of the products of the activity of the cellulolytic enzymes probably escapes immediate fermentation and diffuses into the medium. It seems unlikely that this substance should be glucose, the final product of hydrolytic breakdown of cellulose, for glucose was not attacked by one of the strains. It might well be cellobiose, which was an excellent substrate for both strains.

Carbohydrate utilization

Cellobiose. Both strains grew excellently in the agar medium with 0·1 % cellobiose added instead of filter-paper. In very sparsely inoculated tubes the disk-shaped white colonies often showed a diameter of some 5 mm. after *c.* 8 days growth. Upon transfer to a cellulose medium the ordinary yellow colonies developed again.

Cells of strain S grown on cellobiose agar differed microscopically from the cellulose-grown cells in that the chains were considerably longer, sometimes containing over 100 individuals (Pl. 1, fig. 2). With filter-paper as a substrate, chain length was apparently limited by the size of the fibres. Strain D showed about the same microscopical picture whether grown on cellulose or cellobiose (Pl. 1, fig. 3).

Colonies grown on cellobiose lacked the yellow pigment which is characteristic of growth on filter paper. The following phenomenon illustrates this: when a tube contains both cellobiose (0·1 %) and a strip of filter paper, some of the white colonies in the agar may touch the filter paper as they grow. In these cases one observes that at the place of contact the filter paper turns yellow and from that point the yellow coloration spreads gradually over a limited area of the paper. This is particularly striking in the higher dilutions where the colonies are fewer but much larger.

Glucose. Strain D grew readily in the agar medium with 0·1 % Seitz-filtered D-glucose (British Drug Houses Ltd., Analar). White disk-shaped colonies became visible in the agar, usually 2–3 days after inoculation; they appeared, however, to remain smaller than those grown in presence of cellobiose. Upon transfer to a cellulose medium this latter substance was readily attacked. Strain S did not grow in a 0·1 % glucose medium (Kerfoot's pure bacteriological glucose used).

Other carbohydrates. Strains S and D did not grow on 0·1 % lactose, 0·1 % maltose or 0·5 % starch. Strain D did not attack D-xylose (0·1 %).

Maintenance of cultures

Cellobiose was an excellent substrate for maintenance of the cultures. This was especially so because of the large size of the colonies and the ease with which they could be handled; a few colonies could be sucked out of the agar by means of a Pasteur pipette and blown out into a tube of fresh medium. The original tube could then be re-stoppered after thoroughly gassing the space above the agar with CO_2, and could still be used for other transfers. Many serial transfers were made in cellobiose medium; the ability to attack cellulose was not lost thereby. It was found advisable to transfer the organisms every

7–10 days. When 3- to 4-week old cultures had to be transferred, a very large inoculum was required.

Attempts to improve growth

For strain G it was found accidentally that the colonies became much larger if grown simultaneously with *Cl. sporogenes*. Though less pronounced, this effect was also observed for the strains S and D. The growth-promoting effect of *Cl. sporogenes* was exerted over some distance, since a stab at *c*. 5 mm. from the paper strip was as efficient as a suspension of these bacteria throughout the agar, suggesting that a diffusible factor was involved.

In experiments with strain D proof was obtained that the beneficial effect of *Cl. sporogenes* was due to its adding growth-promoting material to the medium rather than removing an inhibitory substance. When the number of contaminating species had been decreased to 3 or 4, growth of strain D became exceedingly poor. Addition of *Cl. sporogenes* to the medium was very effective in this stage. *Cl. sporogenes* was then grown for 5 days anaerobically on 20 % yeast autolysate; 50 ml. of this culture were centrifuged to remove the bulk of the bacteria and the supernatant was filtered through a Seitz filter. Inclusion of 9 ml. of this sterile filtrate in 50 ml. medium, ± the usual 6 % yeast autolysate, greatly favoured growth of strain D; the colonies grew more quickly and from a more diluted inoculum than without this addition. A pH effect is excluded here, because the culture filtrate had the same pH as the usual medium. The same beneficial effect was obtained when the medium on which *Cl. sporogenes* had grown was sterilized by autoclaving. In this altered medium strain D was readily purified. Once pure, the strain grew well again in the usual medium, the addition of filtrate being no longer essential, though it still had a slightly beneficial effect. Thus *Cl. sporogenes* seems to produce material which is necessary for growth of *Rum. flavefaciens*. *Cl. sporogenes* is not specific in this, and certain other bacteria also have a beneficial effect (Sijpesteijn, 1948). Sufficient of the factor is present in untreated yeast autolysate to allow almost optimal growth of strains S and D in pure culture in a medium with 6 % yeast autolysate; for strain G these conditions seemed to be suboptimal. We may assume that in impure cultures competition of contaminants for this factor sometimes makes an extra supply necessary. When cellobiose was used as a substrate for strain D growth was very good, irrespective of the presence of sterile filtrate of a *Cl. sporogenes* culture.

The yellow pigment accompanying growth on cellulose is much intensified by the presence of *Cl. sporogenes* or sterile culture filtrate. In cellobiose media the colonies appeared white on the usual medium, but in the presence of the *Cl. sporogenes* culture filtrate large colonies appeared slightly yellow.

With regard to the identity of the active material negative results only have been obtained. Experiments with strain G had already shown that addition of a variety of compounds (a mixture of aneurin, riboflavin, nicotinamide, Ca-pantothenate, pyridoxin, *p*-aminobenzoic acid, *i*-inositol, biotin; peptone; Bacto 'Casamino' acids; sterile rumen liquor; cobalt; etc.) did not produce the same effect as *Cl. sporogenes*. The addition of folic acid (Ashe

Laboratories) was tested on strain D during its worst period of growth; 0·01–0·30 μg./6 ml. usual deficient medium gave no better growth. A highly concentrated preparation of vitamin B_{12} (kindly supplied by Dr W. F. J. Cuthbertson, Glaxo Laboratories) used at 0·2 μg. vitamin B_{12}/5 ml. medium showed no growth promotion with strain S.

METABOLIC EXPERIMENTS

The only previous information about the products of cellulose decomposition by *Rum. flavefaciens* was that obtained with strain G in combined culture with *Cl. sporogenes* (Sijpesteijn, 1948). The end-products found in 100 ml. mineral medium + 6 % yeast autolysate + filter-paper + bicarbonate saturated with pure CO_2 as a buffer, were: total volatile acids 4·78 mmol. (namely 0·94 mmol. formic acid, 3·26 mmol. acetic acid and 0·58 mmol. butyric acid), succinic acid 2·41 mmol., lactic acid 1·12 mmol. The amount of filter-paper decomposed was 4·15 mmol. (calculated as glucose).

Two experiments were now carried out with strain S, on cellulose and on cellobiose respectively. No quantitative experiments were carried out with strain D.

Methods of cultivation. The experiments were conducted in a spherical flask with a long neck so that it could be gassed with CO_2 and stoppered. On the lower part of the neck a calibration mark was made to indicate the volume of 130 ml. liquid contents. The medium contained the same salts as mentioned above, with 0·02 % $Na_2S.9H_2O$ instead of Na thiolacetate, 0·5 % $NaHCO_3$, 5 % yeast autolysate (prepared from 1 kg. baker's yeast with 1 l. water) and either a weighed amount of Whatman no. 1 filter-paper cut into pieces, or 0·3 % cellobiose. The liquid sterile ingredients (total volume *c.* 150 ml.) were mixed in a 200 ml. flask by thorough gassing with CO_2 immediately after autoclaving. The fermentation flask containing a little water was autoclaved separately and the water poured out; in the cellulose experiment the paper was autoclaved in this flask. After gassing the flask with CO_2, the medium was poured in to the 130 ml. mark (the remaining volume of medium being kept for estimation of initial reducing value) and the flask re-gassed. The inoculum (not less than *c.* 10 mg. well-attacked filter-paper) was put into the flask, and after further gassing the flask was stoppered and sealed. During the gassing the flask was kept in a water bath at *c.* 40° in order to attain the proper gas pressure.

Usually it took a few days before any indication of growth was visible. In the flask with cellulose, growth started around the piece of paper used as inoculum and gradually spread from there. The flask was stirred periodically to aid attack on other portions of the paper. The incubation period was 13 days for the cellulose and 7 days for the cellobiose experiment; the rate of fermentation probably soon slowed owing to increasing acidity of the medium. After fermentation, the purity of the cultures was checked by anaerobic incubation of a few drops in meat medium and in peptone broth, and by microscopical examination.

Analytical methods. The amount of cellulose fermented was found by estimating the initial and the final dry weight of the paper. The former was computed from the total initial weight of the paper and a dry-weight estimation of a measured sample of it. The inoculum of *c.* 10 mg. paper was added to the initial weight, an error of a few mg. being unavoidable here. At the end of the experiment, the contents of the flask were filtered through a sintered-glass G3 filter to collect the cellulose residue, which was washed on the filter and its dry-weight measured.

The reducing value was estimated before and after fermentation by oxidation with ferricyanide and titration with ceric sulphate (Hassid, 1937). A 5 ml. sample was first treated with 0·5 ml. saturated lead acetate to precipitate protein, and subsequently with 1·0 ml. saturated Na_2HPO_4 to precipitate excess lead acetate. The estimation was carried out on the supernatant after centrifuging. The method was standardized beforehand on recrystallized cellobiose (Light) and glucose (British Drug Houses Ltd., Analar), giving mean values of 2·47 ml. 0·01 N ceric sulphate/mg. cellobiose, and 3·06 ml. 0·01 N ceric sulphate/mg. glucose. The amount of cellobiose decomposed was calculated from the difference of initial and final reducing values of the medium. Succinic acid was estimated manometrically by means of succinic dehydrogenase (Krebs, 1937). Volatile fatty acids were obtained by steam distillation. After destruction of any formic acid by distillation in presence of mercuric sulphate (Friedemann, 1938), chromatograms were made to identify other acids (Elsden, 1946).

No attempt was made to estimate the gaseous metabolic products. Agar cultures showed that only very little gas was produced, part of which must have been due to CO_2 freed from bicarbonate buffer by accumulating acids. The production of any large amounts of metabolic H_2 or CO_2 seems unlikely.

Results. The results obtained are listed in Table 1. The main end-product in terms of carbon recovery was succinic acid; 0·5 mmol. appeared to be produced per mmol. C_6-unit fermented. Propionic and butyric acids were absent. Besides acetic acid, another volatile acid was present which was destroyed in the Friedemann distillation. Though proof is lacking, it seems probable that this was formic acid; in Table 1 the difference between the value for total volatile acids and the titration value after Friedemann distillation is expressed accordingly.

In the experiment with cellulose as substrate there was a slight initial reducing value due to the yeast autolysate present in the medium. After fermentation this value was somewhat higher. Thus a small amount of reducing substance was produced during fermentation. In Table 1 this increase in reducing value is expressed in terms of cellobiose.

The products found do not account for all the carbon fermented. In the pure-culture fermentations, acetic and formic acids represented 23 and 10 % respectively and succinic acid 25-33 %. The latter value depends on whether the CO_2 required in the formation of succinic acid from a C_3-intermediate is entirely supplied as bicarbonate from the medium or whether it can be pictured as metabolic CO_2 that is re-utilized. Changes in the CO_2-content of the medium were not determined.

Table 1. *Products of fermentation of cellulose and cellobiose by* Ruminococcus flavefaciens *strain S*

(Mineral medium with 5 % (v/v) yeast autolysate and 0·5 % (w/v) $NaHCO_3$, saturated with pure CO_2.)

	Cellulose exp.		Cellobiose exp.	
Initial substrate (mg.)	504		433	
Final substrate (mg.)	235		82	
Substrate decomposed (mg.)	269		351	
Substrate decomposed (mmol.)	1·66		1·03	
End-products	mmol.	mmol./mmol. subs.*	mmol.	mmol./mmol. subs.*
Succinic acid	0·85	0·51	1·02	0·50
Total volatile acids	2·14	1·29	2·67	1·31
Formic acid	1·06	0·64	1·2	0·58
Acetic acid	1·08	0·65	1·5	0·73
Reducing sugar	0·07	0·04	—	—

* mmol. subs. = mmol. substrate decomposed expressed in glucose units.

A search for other metabolic products showed that ethanol was absent (method of Conway, 1947). The culture medium gave, however, a weak positive Voges-Proskauer reaction, indicating the presence of small amounts of acetylmethylcarbinol. It seems possible that lactic acid is also an end-product; it was found in the combined culture of strain G with *Cl. sporogenes* (Sijpesteijn, 1948). No lactic acid estimation could be made in the fermentation liquid of the pure culture S. Attempts to estimate the end-products of cellulose decomposition by strain S in a medium buffered with phosphate and gassed with $N_2 + 5\ \%\ CO_2$ were given up because growth was very scanty.

DISCUSSION

Hungate (1950) reported yellow cocci which he isolated from the rumen. Though morphologically these organisms resembled our bacteria, there is a striking difference in the end-products of fermentation. The organisms grown by Hungate for 180 days in a medium with phosphate as buffer gave the following products: 0·307 mmol. CO_2, 0·264 mmol. H_2, 0·55 mmol. acetic acid, 0·252 mmol. ethanol, 0·68 mmol. lactic acid, traces of succinic acid; 80·0 mg. cellobiose (= 0·234 mmol.) accumulated and 259·6 mg. cellulose (= 1·60 mmol. calculated as glucose) disappeared. Essentially the same figures were found in a second experiment, which lasted 67 days. These results differ greatly from the present ones, but it must be stressed that the two sets of experiments were conducted under very different conditions with regard to the availability of CO_2. It seems likely that Hungate's experiment was run without addition of CO_2, whereas the present experiments took place in a bicarbonate medium saturated with CO_2. These different conditions may perhaps account for the observed qualitative and quantitative differences in the end-products. Certainly it seems not unlikely that the CO_2 pressure affects the yield of succinic acid and thus of other products. Unfortunately our attempts to investigate this failed. Very little information is available on the effect of the

CO_2 pressure on the composition of the end-products of different bacteria. Johns (1949), with *Veillonella gazogenes*, found that the CO_2 pressure greatly influenced the yield of propionic acid, a decarboxylation product of succinic acid, in a lactate medium. On the other hand, the figures of Hungate (1950) for *Bacteroides succinogenes* show only a very slight effect. Thus it is not possible to decide at present whether the yellow coccus with which Hungate worked is identical with *Rum. flavefaciens*.

With regard to the fate of the observed end-products in the rumen the following may be said. In the rumen the CO_2 pressure is usually high (*c.* 35–70 % partial pressure) so that the fermentation products should be similar to those found in the present experiments. Recent investigations have shown that succinic acid is rapidly decarboxylated in the rumen to propionic acid (Elsden & Sijpesteijn, 1950) which is not further metabolized. Like the acetic acid found in the present experiments, propionic acid accumulates in *in vivo* and *in vitro* fermentations of cellulose by the rumen population (Elsden, 1945; Marston, 1948). From the rumen these acids directly enter the blood stream of the animal and undergo further conversions in its tissues (cf. Elsden & Phillipson, 1948). It seems unlikely that the third metabolic product, formic acid, is stable in the rumen, but the mode of its transformation there has not yet been cleared up. Lactic acid may be one end-product of cellulose decomposition by *Rum. flavefaciens*; it is converted in the rumen into propionic acid and acetic acid (Elsden, 1945).

The fact that one of the present strains (S) does not grow in a glucose medium but can do so in cellulose and cellobiose media, is noteworthy. A similar observation has been made for other cellulose-decomposing bacteria (Hungate, 1947, 1950; McBee, 1948, 1950). Stanier (1942) and Enebo (1949) reported cellulose-decomposing organisms which attacked cellulose and cellobiose at much greater rates than they did glucose.

CLASSIFICATION

In 1948 the author proposed a new genus, *Ruminococcus*, in the tribe Streptococceae of the family Micrococcaceae according to the classification of Kluyver & van Niel (1936). The name *Ruminococcus flavefaciens* was proposed for the present species. The description of the genus and the species follows here including some additional data.

Description of the genus Ruminococcus

Spherical cells; Gram-positive; generally in chains or pairs; non-motile; no endospores. Cellulose and other carbohydrates fermented with production of large amounts of succinic acid. Anaerobic.

The type species is *Rum. flavefaciens*.

Description of the species Ruminococcus flavefaciens

Morphology: cells almost spherical, diameter 0·8–0·9 μ.; generally in chains or pairs; a yellow pigment is produced on cellulose. Utilizable carbon sources:

cellulose, cellobiose, glucose used by certain strains; maltose, lactose, xylose, and starch not used. Catalase-negative. Obligate anaerobe. Mesophilic.

Source and habitat: occurs in vast numbers in rumen of cattle and sheep, and probably also in that of other ruminants and in caecum and colon of herbivorous mammals.

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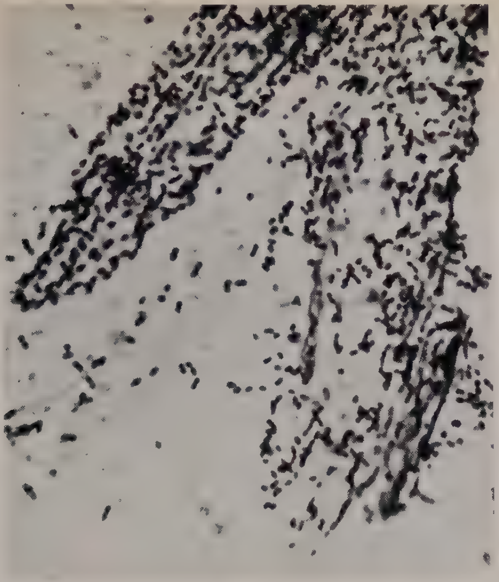

Fig. 1

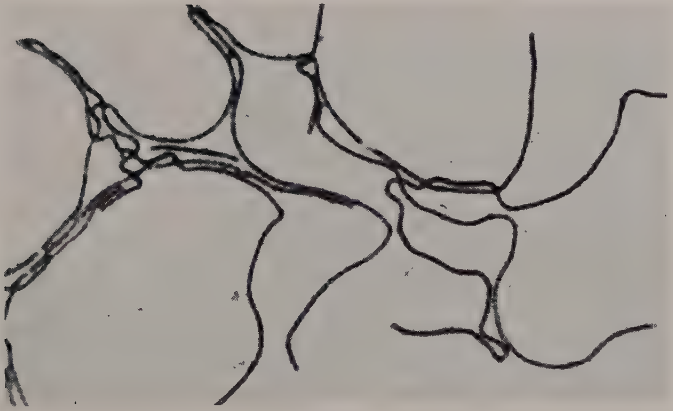

Fig. 2

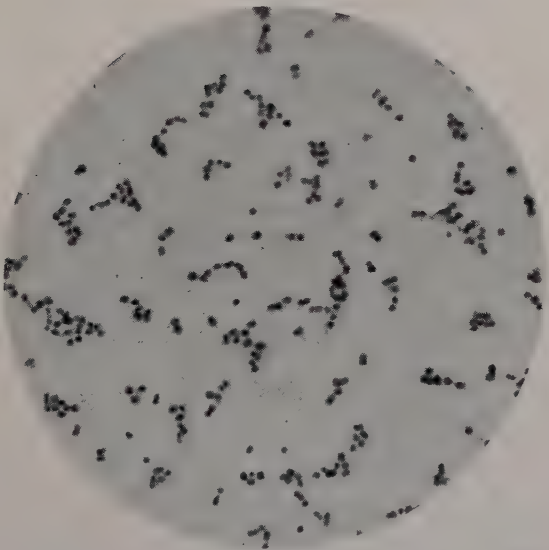

Fig. 3

A. K. Sijpesteijn—On *Ruminococcus flavefaciens*. Plate 1

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EXPLANATION OF PLATE

Fig. 1. *Ruminococcus flavefaciens* strain D on filter-paper fibres grown in agar medium for 5 days. Methyl violet stain; × 1000.

Fig. 2. *Rum. flavefaciens* strain S; from 0·1 % cellobiose agar after 4 days. Gram stain; × 1000.

Fig. 3. *Rum. flavefaciens* strain D; from 0·1 % cellobiose agar after 6 days. Methyl violet stain; × 1000.

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Nutritional Requirements of *Bacterium coli* at 44°

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SUMMARY: A strain of *Bacterium coli* capable of growth in broth and in an ammonium + glucose + salts medium at 37° and in broth at 44°, died in the defined medium at 44°. The addition of glutamic acid to this medium allowed the growth temperature to be raised slightly; the further addition of nicotinamide allowed a further increase in growth temperature.

One of the criteria on which Public Health bacteriologists rely to identify strains of *Bacterium coli* as being of faecal origin is the ability to ferment lactose with the evolution of gas in a bile salt medium at 44°. The experiments recorded in this paper show that ability to multiply at 44° depends not only on the strain of *Bact. coli* but also on the nutritive materials supplied.

METHODS

The defined medium used throughout the work was Bigger's (1950) modification of Gale's (1947) medium which contains only organic salts and glucose, the sole nitrogen source being ammonia. Broth, where used, contained 1 % Lab-Lemco and 1 % peptone. Experiments were carried out with 5 ml. volumes of medium in small glass bottles covered with loosely fitting aluminium caps. When other substances were required in the medium, additions were made either in bulk or by adding to the individual bottles a sufficient number of drops (Dreyer pipette) of a suitable dilution of the substance. Serial dilutions, to halve the concentration of a substance, were made by transferring 5 ml. amounts from bottle to bottle. *Bact. coli* strain no. 18 was used as test organism, except in certain experiments designed to determine whether the phenomenon described occurred with other strains of *Bact. coli.* The cultural characteristics of *Bact. coli* strain no. 18, which was isolated from faeces, are given in Table 4. Inoculation was, in all cases, carried out by adding to 5 ml. of the medium, one drop (Dreyer pipette) of a 1/10,000 dilution in buffered saline of the test organism grown in defined medium at 37°. Cultures were examined every 24 hr. during incubation. One 5 mm. loopful of each culture which had remained clear was spread over the surface of an agar slope, which was examined after 24 hr. incubation, to determine the colonies resulting from the viable organisms in the original culture.

RESULTS

Growth curves were obtained from viable counts (2 hr. intervals) on *Bact. coli* growing in defined medium or in broth at 37 and 44°. It was found that whereas the organism multiplied freely in both media at 37° and in broth at 44°, it died when exposed to 44° in defined medium. The effect of adding certain of the broth constituents to defined medium was then examined. A concentration of 25 mg. Lab-Lemco/100 ml. defined medium was sufficient to allow full multiplication of *Bact. coli* at 44°; whilst as little as 1 part in 128,000 allowed some organisms to survive; at lower concentrations of Lab-Lemco the bacteria died. The protective effect of peptone was similar to that of Lab-Lemco but slightly less marked. Lab-Lemco and peptone were not the only substances which showed the 'protective effect'; various meat and vegetable extracts and gelatin acted similarly. A mixture containing nineteen pure substances some of which, it was thought, might be responsible for the effect was tested. By a series of grouping experiments, in which inactive constituents of the mixture were eliminated one by one, it was found that full protection was afforded by adding to defined medium glutamic acid at 1/4000 and asparagine, nicotinamide, or nicotinic acid. In the presence of glutamic acid (1/500) a concentration of 1/500 asparagine was required for growth. Nicotinamide was much more active, a dilution of 1/81,000,000 allowing visible turbidity to develop in 24 hr.; nicotinic acid had the same activity.

Table 1. *The effect of incubation at* 52° *on a suspension of* Bact. coli *strain no.* 18 *in defined medium with and without glutamic acid + nicotinamide*

(Samples (loopful) of the various media were taken at intervals and plated.)

Period of incubation (min.)	Surviving organisms per loopful	
	Defined medium	Defined medium + glutamic acid (1/1000) + nicotinamide (1/10⁷)
0	+++	+++
10	+++	+++
20	+	+
30	216	200
40	57	87
50	43	44
60	40	28

Where colonies were too numerous to count the results were recorded:

+ Uncountable number of discrete colonies.
+++ Homogeneous confluent growth.

Table 1 shows the result of an experiment to determine the thermal death times for *Bact. coli* in defined medium and in defined medium with added glutamic acid (1/1000) and nicotinamide ($1/10^7$) at 52°. No significant difference between the thermal death times in the two media was found.

The rates of growth of *Bact. coli* strain no. 18 in defined medium with various additions were examined, and are summarized in Table 2. A slight increase was noticeable in the rate of multiplication at 37° where glutamic acid and nicotinamide were both present, but not with nicotinamide alone, where there was slight retardation at both temperatures.

To determine whether substances capable of sustaining growth at 44° were liberated into the medium during growth at 37°, defined medium (50 ml.) was inoculated with *Bact. coli* strain no. 18 and incubated for 24 hr. at 37°. The culture was then filtered through a Seitz EK filter, boiled briefly and the

Table 2. *Viable counts of* Bact. coli *strain no.* 18 *after different periods of incubation* (*at* 37° *or* 44°) *in four defined media*

(Media: A = basal defined medium alone;
B = basal defined medium + glutamic acid (1/1000);
C = basal defined medium + nicotinamide ($1/10^7$);
D = basal defined medium + glutamic acid (1/1000) + nicotinamide ($1/10^7$).

	Incubated at 37°				Incubated at 44°			
Medium ...	A	B	C	D	A	B	C	D
Period of incubation (hr.)	Plate counts (colonies/loopful)							
0	34	34	34	34	34	34	34	34
2	61	42	38	55	24	31	36	42
4	120	81	80	193	23	23	9	58
6	+	+	193	++	30	18	8	180
8	++	++	+	+++	26	17	11	+
10	+++	+++	++	+++	22	14	8	+++
12	+++	+++	+++	+++	13	8	0	+++

Where colonies were too numerous to count the results were recorded:
+ Uncountable number of discrete colonies.
++ Confluent growth with individual colonies discernible.
+++ Homogeneous confluent growth.

pH adjusted to 7·5. Halving dilutions of this filtrate plus glutamic acid (1/1000) or nicotinamide ($1/10^7$) were inoculated with *Bact. coli* no. 18 and incubated at 44°. Growth occurred only in the series to which glutamic acid had been added, indicating that material had been produced during growth at 37° which, when present with glutamic acid, allowed growth at 44°. This material may well have contained nicotinic acid or a related substance, synthesized by the *Bact. coli* grown at 37°.

Table 3. *Protective effect of nicotinamide and glutamic acid on five strains of* Bact. coli *grown in defined medium for* 24 hr. *at various temperatures*

(Media: A = defined medium alone;
B = defined medium + 1/1000 glutamic acid;
C = defined medium + 1/1000 glutamic acid + $1/10^7$ nicotinamide.)

	Strain of *Bact. coli*														
	No. 2			No. 7			No. 12			No. 13			No. 18		
	Medium														
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Growth temp (° C.)	Relative degree of growth														
37	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT
41	C	T	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT
43	C	T	TT	C	TT	TT	C	TT	TT	C	TT	TT	C	T	TT
44	C	C	TT	C	TT	TT	C	TT	TT	C	T	TT	C	C	TT
45	C	C	C	C	C	T	C	C	TT	C	C	C	C	C	T

Naked eye appearance of culture recorded: C, clear; T, slight turbidity; TT, marked to full turbidity.

Nicotinamide and glutamic acid are both required for growth at 44°, but at 42–43°, although still unable to grow in the basal defined medium, *Bact. coli* strain no. 18 grew when only glutamic acid was added to the medium. Table 3 shows the effects of various temperatures on five strains of *Bact. coli* in the different media. Although the temperature at which a given additional nutrient is required differs slightly for the different strains, no organism grew at 44° without some addition to the medium. The addition of glutamic acid increased the temperature at which a given strain could grow, whilst the further addition of nicotinamide increased still further the tolerated temperature. Of the strains of *Bact. coli* tested, all were able to grow in the basal defined medium at 37° and none grew in that medium at 44°; but these were able to grow at 44° after the addition of glutamic acid and nicotinamide. The characters of the strains of *Bact. coli* tested are summarized in Table 4.

Table 4. *The characters shown in the five strains tested*

Strain no.	Fermentation of		Growth on Koser's medium	Indole production	Voges-Proskauer reaction	Motility
	Dulcitol	Saccharose				
2	AG	–	–	+	–	–
7	AG	–	–	+	–	–
12	–	AG	+	–	+	–
13	A	–	–	–	–	+
18	–	AG	–	+	–	+

A = acid; AG = acid and gas.

DISCUSSION

The mechanism of this protective action is probably not directly physical; if it were it would be accompanied, at higher temperatures, by an increase in the thermal death time of the organisms tested under the same test conditions. Table 2 shows that death of a culture was not sudden, although often complete within 24 hr. It may be suggested that death was the result of an inability of the organisms in the inoculum to multiply, the organisms dying slowly as they usually do under conditions unfavourable to normal division. The slightly greater rate of growth at 37°, when both glutamic acid and nicotinamide were present, was probably due to a nutrient being present in a more completely prepared form, thus requiring less synthetic activity on the part of the organism. This slight stimulating effect by itself appears inadequate to explain the phenomenon of growth at 44°. For the production of essential metabolites required by the organism from the inorganic salts, ammonia and glucose of the defined medium, numerous series of enzymic syntheses are required. The breaking of any step in the biosynthesis of the essential metabolite nicotinamide would prevent growth. It seems, therefore, possible that at 44° an enzyme responsible for one stage in the biosynthesis of nicotinamide is inactivated and, in consequence, the organism becomes dependent upon an external supply of nicotinamide or nicotinic acid.

Glutamic acid could be regarded as playing a similar role in other biosyntheses. Table 3 shows that glutamic acid was required in general at a lower temperature than nicotinamide, but that the actual temperature at which it became an essential nutrient was a function of the particular strain, as it was with nicotinamide.

Certain practical aspects of these findings may be considered as they effect the investigations usually employed to distinguish faecal strains of *Bact. coli*, which are described as able to grow at 44°, from non-faecal strains which cannot grow at this temperature. As ability to grow at a specified temperature may be a function not only of the strain of organism, but also of the constituents of the medium in which it is grown, it is evident that a medium deficient in nicotinamide might lead to the identification of a faecal *Bact. coli* as a non-faecal type. Although glutamic acid and nicotinamide produce a protective effect against an otherwise adverse temperature, it cannot be asserted that these substances are the only ones which are responsible for the effect with complex preparations such as Lab-Lemco.

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Tetraploid *Saccharomyces*

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SUMMARY: A yeast hybrid heterozygous for many alleles produces an occasional ascus in which instead of the expected 2 dominant:2 recessive segregations one finds 4 dominant:0 recessive ratios for most of the characters. The single ascospore cultures are often incapable of copulating with either mating type. Genetical analysis has revealed that such asci contain ascospores which are diploid and heterozygous for the mating type alleles and other characters. The single ascospore cultures, heterozygous for mating type, may sporulate and by characterization of their progeny the genotype of the ascospore can be determined. Other diploid cultures originating from single ascospores are homozygous for mating type. Cells from these cultures (unlike the diploid cells heterozygous for mating type) are capable of mating, and when mated with similar cells of complementary mating type produced tetraploid yeasts. Triploids were produced by mating diploids with haploids. The tetraploid segregates to produce the expected tetrad types and gametic ratios. Some sporulating cultures produced unexpected types of asci for characters controlling sugar fermentation but interpretation of their significance is not feasible since our customary control of breeding procedure does not apply in the analysis of diploid cultures which sporulate on ordinary agar slants.

METHODS

Safeguards in the breeding procedure. We have developed a breeding procedure which excludes errors due to intervening sporulations and copulations between the generations which are analysed. In the earlier phases of this work single ascospore cultures which produced spores were not unusual (Lindegren & Lindegren, 1944). A rigid programme of selection was established and sporulating haplophase cultures were never used as parent breeding stocks. Diplophase cultures were never maintained as sources for breeding stocks since sporulation would be expected to occur and would quickly render them heterogeneous. Non-sporulating single ascospore haploid cultures were preferred as breeding stocks, since a minimum of variation occurs when they are carried on a rich nutrient medium by heavy transfers. A mating is made by mixing two heavy inocula of these cultures in a small amount of broth and transferring the cells to presporulation agar as soon as zygotes appear. On the presporulation agar, growth of both haplophase and diplophase occurs but spores are not produced. Most of the cells from the presporulation agar are transferred to gypsum. The immediate descendants of the zygotes sporulate while the haplophases do not. Each haploid culture used in a mating is carefully tested for its ability to sporulate by growth on presporulation agar followed by transfer to gypsum. Those which show any tendency to sporulate under these conditions are not used.

The advantages of our procedure are as follows: (1) Non-sporulating strains (haplophases) constitute the breeding stocks. (2) The composition of the presporulation medium permits the zygotes to multiply in the absence of sporulation. (3) The asci are formed on gypsum where germination of the spores does not occur and, since they are picked directly from the gypsum and dissected, intervening copulations are excluded. (4) The control of the time factor in this process may be as important an advantage as any other. Copulations begin about 6 hr. after the mixtures are made and the hybrids are complete after 12 hr. After 24 hr. on presporulation agar, which has been designed especially to hasten sporulation, the cells are transferred to gypsum and sporulate within 12 hr. Many current procedures require 10 days or 2 weeks to produce spores, and the disadvantage of such an extended period lies in the fact that critical observation cannot be maintained and therefore control of the procedure is lost.

It should be pointed out that sporulation of the diploid culture followed by copulation between spores or gametes could quickly lead to a new genotype. In such a case if only a few markers are used, the ratio of asci containing four dominants to those containing a fewer number might be nothing more than an evidence of the frequency with which intervening copulations had occurred. Assurance against this type of accident may be obtained by employing genetical stocks with large numbers of markers. Our current breeding stock (Lindegren & Lindegren, 1951) is heterozygous for genes marking seven loci controlling sugar fermentation, six loci controlling B vitamin synthesis, eight loci controlling amino-acid synthesis, two loci controlling adenine synthesis and two controlling uracil synthesis.

Tetrad analysis is the procedure which gives the fungi a special advantage in genetical study. In the genetics of higher organisms, the events occurring during the reduction division must be inferred on the basis of the distribution of the various genetical characteristics among the progeny of a mating. For example, segregation of chromosomes in the sperm mother cell of a diploid male is inferred from the characteristics displayed by his offspring after mating to one or many females. In yeast, such inference is unnecessary since the four spores which are the homologues of the four sperm can be separated and grown to maturity as individual clones. Furthermore, the recovery among the four spores from a single ascus of characters originating from both parents is unequivocal proof of the hybrid nature of the diploid cell from which the ascus was produced.

RESULTS

The present paper analyses a type of deviation from the expected 2:2 Mendelian segregation in *Saccharomyces* asci due to the spontaneous diploidization of the ascospores. This phenomenon has also been described by Roman, Hawthorne & Douglas (1951). In addition, it reports the controlled production of triploid and tetraploid hybrids by the use of diploid gametes originating from diploid ascospores homozygous for mating type.

Spontaneous diploidization of ascospores. The analysis of hybrid 10868 × 11056 (Table 1) resulted in the discovery of an exceptional ascus in which most of the segregants showed the dominant phenotype. 10868 was a white and 11056 was a pink (adenine-dependent) culture. Two of the twenty-seven asci were

Table 1. *Analysis of a hybrid heterozygous for ten loci in which one ascus containing spontaneously diploid ascospores occurred*

Culture no.											
10868	α	*SU*	*G*	*MA*	*me*	*MG*	*W*	*AD*	*ly*	*py*	*UR*
						by					
11056	*a*	*su*	*g*	*ma*	*ME*	*mg*	*P*	*ad*	*LY*	*PY*	*ur*
11275	*a*	*su*	*g*	*MA*	*me*	*MG*	*P*	*ad*	*LY*	*py*	*UR*
11276	α	*SU*	*G*	*ma*	*ME*	*mg*	*W*	*AD*	*LY*	*PY*	*ur*
11277	α	*su*	*G*	*ma*	*ME*	*mg*	*W*	*AD*	*ly*	*py*	*ur*
11278	*a*	*SU*	*g*	*MA*	*me*	*MG*	*P*	*ad*	*ly*	*PY*	*UR*
11294	*a*	*SU*	*g*	*MA*	*me*	*MG*	*W*	*AD*	*LY*	*PY*	*UR*
11295	?	*SU*	*G*	*MA*	*ME*	*MG*	*W*	*AD*	*LY*	*PY*	*UR*
11296	α	*SU*	*G*	*MA*	*ME*	*MG*	*W*	*AD*	*LY*	*py*	*UR*
11297	?	*SU*	*G*	*MA*	*ME*	*mg*	*W*	*AD*	*LY*	*PY*	*UR*

The mating type genes in yeast have been designated respectively a and α. Cultures of mating type a are able to copulate with α, but a cultures are not able to copulate with a, nor is α able to copulate with α. Each regularly segregating ascus normally contains two a and two α spores. The diploid cultures (a/α) produced by copulations between a and α cells are incapable of copulating with either mating type and cannot be classified with regard to mating type. Further analysis revealed that 11295 and 11297 were a/α; (?) indicates their inability to copulate with a known tester.

The letters *SU*, *G*, *MA*, *ME*, *MG* indicate the ability of the cultures to produce gas in Durham tubes of nutrient medium which contain respectively: sucrose, galactose, maltose, melibiose or α-methylglucoside as the sole carbohydrate sources. The corresponding small letters indicate their inability to produce measurable amounts of gas under these circumstances. The letters *AD*, *LY*, *UR*, *PY*, indicate the ability of the cells to grow in Burkholder's medium (Burkholder & Moyer, 1943) lacking the following nutrilites, respectively: adenine, lysine, uracil and pyridoxin. The small letters indicate inability to grow under these circumstances. The *ad* cultures are pink (*P*).

exceptional. One ascus produced three pink and a single white culture, while the other (11294–11297) produced four white cultures, all of which were adenine-independent. Ascus 11275–11278 is one of a sample of five asci which were characterized for all ten loci and which showed regular segregation. The other asci were merely characterized for pink (*P*) and white (*W*) with the results shown below:

P	*P*	*W*	*W*	17
P	*P*	*W*	—	2
P	—	*W*	*W*	5
P	*W**	*W*	*W*	1
P	*P*	*P*	*W*	1
W	*W*	*W*	*W*	1

Among the asci in which only three spores survived no exceptions were encountered, although it is obvious that this does not establish them as standard asci. Considerable variation from light pink to obvious pink was observed so that it is possible, especially in the case of the white culture marked with an asterisk, that the colour was too light to be clear, or that an overgrowth of white cells may have obscured the pink phenotype. The reverse of this situation has not occurred. Under our conditions a genetically white culture does not take on a pink appearance.

Work in this laboratory has consistently confirmed the relation between pink colour and adenine or hypoxanthine dependence. Pink cultures have always proved to be adenine-dependent, and occasional cultures which were pink or apparently white, may also be adenine-dependent, but no adenine-independent culture is ever pink. In yeast (as in *Neurospora*) adenine-dependent (*ad*) cultures are pink owing to the accumulation and apparent polymerization of a precursor of adenine. Ability to complete the synthesis of adenine results in the removal of the precursor and the absence of the pink colour. Hybrids heterozygous for *AD*/*ad* are white. The fact that one parent was pink and the other white suggested that the four spores of ascus 11294-11297 were diploid and heterozygous for *AD*/*ad*. The appearance of pyridoxin-independent, lysine-independent and melibiose fermenters in this ascus clearly demonstrates that it is not an illegitimate offspring of 10868.

Table 2. *Phenotypes of the ascospores derived from 4-spored asci of culture 11295 SU G MA MG W AD* (*only three spores survived in the asci*)

Culture no.											
12260	α	*SU*	*g*	*MA*	*me*	*mg*	*P*				
12261	*a*	*SU*	*g*	*MA*	*ME*	*MG*	*W*				
12262	*a*	*SU*	*G*	*MA*	*me*	*MG*	*W*				
12263	α	*SU*	*G*	*MA*	*me*	*mg*	*W*				
12264	*a*	*SU*	*g*	*MA*	*me*	*mg*	*P*				
12265	α	*SU*	*g*	*MA*	*ME*	*MG*	*P*				
12316		*SU*	*g*	*MA*	*ME*	*mg*	*P*	*ad*	*ly*	*py*	*ur*
12317		*SU*	*G*	*MA*	*ME*	*mg*	*P*	*ad*	*ly*	*py*	*UR*
12318		*SU*	*G*	*MA*	*me*	*MG*	*W*	*AD*	*ly*	*py*	*ur*
12319		*SU*	*g*	*ma*	*me*	*mg*	*P*	*ad*	*LY*	*PY*	*UR*
12320		*SU*	*g*	*ma*	*ME*	*mg*	*P*	*ad*	*ly*	*py*	*ur*
12321		*su*	*G*	*MA*	*ME*	*MG*	*W*	*AD*	*LY*	*PY*	*UR*
12322		*SU*	*G*	*MA*	*me*	*mg*	*W*	*AD*	*ly*	*py*	*ur*
12323		*SU*	*g*	*MA*	*me*	*mg*	*W*	*AD*	*LY*	*PY*	*UR*
12324		*SU*	*g*	*ma*	*ME*	*MG*	*P*	*ad*	*LY*	*PY*	*UR*
12325		*SU*	*g*	*ma*	*me*	*MG*	*P*				
12326		*SU*	*G*	*MA*	*ME*	*MG*	*W*				
12327		*SU*	*G*	*MA*	*ME*	*mg*	*P*				

For explanation of symbols see Table 1.

Segregation of a diploid culture obtained from a single ascospore. Culture 11295 sporulated on the agar slant without recourse to either presporulation agar or gypsum. Six 4-spored asci (only three spores germinated from each) were analysed (Table 2). It should be emphasized that under these conditions our rigid control of the genetical method does not apply. If copulations occur on the slant the mixture of *MA MA MA*—and *ma ma MA*—asci could have alternative interpretations; if the diploid cells were *MA*/*ma* an intervening sporulation could have produced a copulation between two *MA* segregants producing the *MA MA MA MA* asci. The appearance, however, of any recessive alleles is evidence supporting the view that the original culture was heterozygous. On this basis 11295 was heterozygous for all ten loci with the possible exception of *SU*/*su*.

Table 3. *Phenotype of ascospores derived from 4-spored asci of culture* 11297. *SU G MA ME mg* (*in two asci only three spores survived*)

Culture no.											
12270	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*	*AD*	*LY*	*PY*	*ur*
12271	*α*	*su*	*G*	*MA*	*ME*	*mg*	*P*	*ad*	*ly*	*py*	*UR*
12272	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*	*AD*	*ly*	*PY*	*ur*
12273	*α*	*SU*	*G*	*MA*	*ME*	*mg*	*P*	*ad*	*LY*	*py*	*UR*
12274	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*	*AD*	*LY*	*PY*	*UR*
12275	*α*	*su*	*G*	*MA*	*ME*	*mg*	*P*	*ad*	*ly*	*py*	*ur*
12276	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*	*AD*	*LY*	*PY*	*UR*
12277	*α*	*SU*	*G*	*MA*	*ME*	*mg*	*W*	*ad*	*ly*	*py*	*ur*
12278	*a*	*su*	*g*	*MA*	*ME*	*mg*	*W*				
12279	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*				
12280	*α*	*SU*	*G*	*MA*	*ME*	*mg*	*P*				
12281	*α*	*SU*	*G*	*MA*	*ME*	*mg*	*P*				
12282	*α*	*SU*	*g*	*MA*	*ME*	*mg*	*P*				
12283	*α*	*su*	*g*	*MA*	*ME*	*mg*	*P*				
12284	*a*	*SU*	*G*	*MA*	*ME*	*mg*	*W*				
12285	*a*	*SU*	*G*	*MA*	*ME*	*mg*	*W*				
12286	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*				
12287	*α*	*su*	*G*	*MA*	*ME*	*mg*	*W*				
12288	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*P*				
12289	*a*	*SU*	*g*	*MA*	*ME*		*W*	*AD*	*LY*	*PY*	*ur*
12290	*a*	*SU*	*G*	*MA*	*ME*		*P*	*ad*	*LY*	*PY*	*ur*
12291	*α*	*SU*		*MA*	*ME*		*P*	*ad*	*ly*	*PY*	*UR*

For explanation of symbols see Table 1.

Culture 11297 was also analysed (Table 3); and the same arguments apply. Six 4-spored asci (in two only three spores germinated) were cultured. Irregularities were encountered in the SU/*su* segregation, but this phenomenon requires further study, since: (1) the dominant *SU* parent arose from a pedigree in which irregular segregation of the ability to ferment sucrose occurred; (2) Hestrin & Lindegren (1950) have shown that sucrase may be a concealed enzyme which becomes overt on special occasions, and Gilliland (1949) has shown that at least three different alleles may control sucrose fermentation.

Culture 11297 appears to be heterozygous for *a*/*α*, *SU*/*su*, *G*/*g*, *AD*/*ad*, *LY*/*ly*, *PY*/*py* and *UR*/*ur*, and homozygous for *MA*, *ME* and *mg*.

These facts are consistent with the view that the ascospores in ascus 11294–11297 are diploid. Cultures 11294 and 11296 do not sporulate but the genotypes, shown below, may be inferred from the data obtained from 11295 and 11297.

11294	*a*/*a*	*SU*/*su*	*g*/*g*	*MA*/?	*me*/*me*	*MG*/?	*AD*/*ad*	*LY*/*ly*	*PY*/*PY*	*UR*/*ur*
11295	*a*/*α*	*SU*/*su*	*G*/*g*	*MA*/*ma*	*ME*/*me*	*MG*/*mg*	*AD*/*ad*	*LY*/*ly*	*PY*/*py*	*UR*/*ur*
11296	*α*/*α*	*SU*/*su*	*G*/*G*	*MA*/?	*ME*/*me*	*MG*/?	*AD*/*ad*	*LY*/*ly*	*py*/*py*	*UR*/*ur*
11297	*a*/*α*	*SU*/*su*	*G*/*g*	*MA*/*MA*	*ME*/*ME*	*mg*/*mg*	*AD*/*ad*	*LY*/*ly*	*PY*/*py*	*UR*/*ur*

When all four diploid spores are of the dominant phenotype, as is the case with *SU*, *MA*, *AD*, *LY*, *UR* the cultures may all be heterozygous. Culture 11294 is apparently *g*/*g*; cultures 11295 and 11297 are *G*/*g*, suggesting that 11296 is *G*/*G*.

Since all four members of the tetrad are diploid the change must have occurred preceding meiosis.

Production of a triploid and tetraploid zygotes

Culture 11294 has been inferred to be a/a and 11296 to be α/α; these cultures are therefore diploid gametes of contrasting mating type. They were mated with each other and with haploid gametic clones. When mated to haploids, triploid cells were produced and when mated with each other they produced tetraploid cells (Fig. 1). The cells of triploid and tetraploid cultures are large compared with those of standard diploid and haploid cultures and with diploid gametes.

Large yeast cells have been obtained by other workers following treatment with a variety of agents, and it has been suggested that the large cells arose through polyploidy (Bauch, 1942; Thaysen & Morris, 1943). Duraiswami & Subramaniam (1950) have argued, however, that cell-size is not an adequate criterion for the identification of polyploid series in yeast. They have proposed that fluctuations can be identified by the appearance of the colonies and by variations in the number of chromatic particles found within the cell. Duraiswami & Subramaniam's cytological data concerning chromatic particles do not support the view that changes in ploidy occur, since they did not locate the particles with regard to either the centrosome, the nuclear vacuole, or the cytoplasm. The locations of the Feulgen-positive structures in the yeast cell are discussed in detail by Lindegren & Rafalko (1950). The controlled production of triploid and tetraploid yeasts should not be taken as supporting Subramaniam's views on variations of ploidy; the inadequacy of his views has been exposed by Winge (1951) in a recent critique.

Tetrad analysis of the tetraploid

The tetraploid hybrid between 11294 and 11296 was analysed (Table 4). The general excess of dominant characteristics among the segregants from the tetraploid is expected. The hybrid is *AD AD ad ad*. The *ad* cultures are pink (*P*) and the *AD* cultures are white (*W*). Segregations for *P* and *W* are not in contradiction of expectation. The zygote is presumed to be *ME me me me*. On this basis the third ascus is not explicable unless an intervening sporulation may have occurred on the agar slant, a possibility which cannot be excluded in dealing with cultures which sporulate on the agar slant.

Tetrad analysis of diploid segregants from the tetraploid

The cultures of the first three asci were tested for their ability to sporulate with the results indicated in Table 4. Single ascospore cultures from 12369 and 12370, both of which were *me*, yielded only *me* offspring (Table 5). Culture 12380 was a fermenter of galactose. An excess of *g* ascospores occurred in four asci. The third ascus from 12380 was *me me me me*. The parent culture was a melibiose fermenter. Culture 12380 which arose as a segregant from the tetraploid zygote exhibited the expected segregation of *P* and *W*, but the segregations of fermentative ability toward galactose and melibiose were exceptional. Exceptions in this case may, or may not, indicate exceptions to Mendelian rules, since, as has been pointed out, our customary control over intervening life cycles is not feasible when the diploid cultures sporulate on agar slants.

Table 4. *Segregation of the tetraploid* (11296 × 11294)

Culture no.								Spores
12369	?	*SU*	*G*	*MA*	*me*	*MG*	*W*	+
12370	?	*SU*	*G*	*MA*	*me*	*MG*	*W*	+
12371	?	*SU*	*G*	*MA*	*ME*	*MG*	*W*	+
12372	?	*SU*	*G*	*MA*	*me*	*MG*	*P*	−
12373	*α*	*SU*	*G*	*MA*	*ME*	*MG*	*W*	−
12374	*α*	*su*	*g*	*MA*	*ME*	*MG*	*P*	−
12375	*a*	*su*	*G*	*MA*	*me*	*MG*	*W*	−
12376	*a*	*SU*	*g*	*MA*	*me*	*MG*	*W*	−
12377	?	*SU*	*G*	*MA*	*ME*	*MG*	*P*	−
12378	?	*SU*	*G*	*MA*	*me*	*MG*	*W*	−
12379	?	*SU*	*G*	*MA*	*ME*	*MG*	*W*	+
12380	?	*SU*	*G*	*MA*	*ME*	*MG*	*W*	+
12381		*SU*	*G*	*MA*	*ME*	*MG*	*W*	
12382		*SU*	*G*	*MA*	*me*	*MG*	*W*	
12383		*SU*	*G*	*MA*	*me*	*MG*	*W*	
12384		*SU*	*g*	*MA*	*ME*	*MG*	*P*	*ad*
12385		*SU*	*G*	*MA*	*me*	*MG*	*W*	*AD*
12386		*SU*	*G*	*MA*	*ME*	*MG*	*W*	*AD*
12387		*SU*	*g*	*MA*	*ME*	*MG*	*P*	*ad*
12388		*SU*	*G*	*MA*	*me*	*MG*	*W*	*AD*
12389		*SU*	*G*	*MA*	*ME*	*MG*	*W*	*AD*
12394		*SU*	*G*	*MA*	*me*	*MG*		
12395		*SU*	*G*	*MA*	*ME*	*MG*		
12396		*SU*	*G*	*MA*	*ME*	*MG*		

For explanation of symbols see Table 1.

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Table 5. *Tetrad analyses of three diploid ascospore cultures derived from the tetraploid*

Culture 12369: *S G MA me MG W* spores

Culture no.								
12404	*a*	*SU*	*G*	*MA*	*me*	*MG*		
12405	*α*	*SU*		*MA*	*me*	*MG*		
12406	*α*	*SU*	*G*	*MA*	*me*	*MG*		

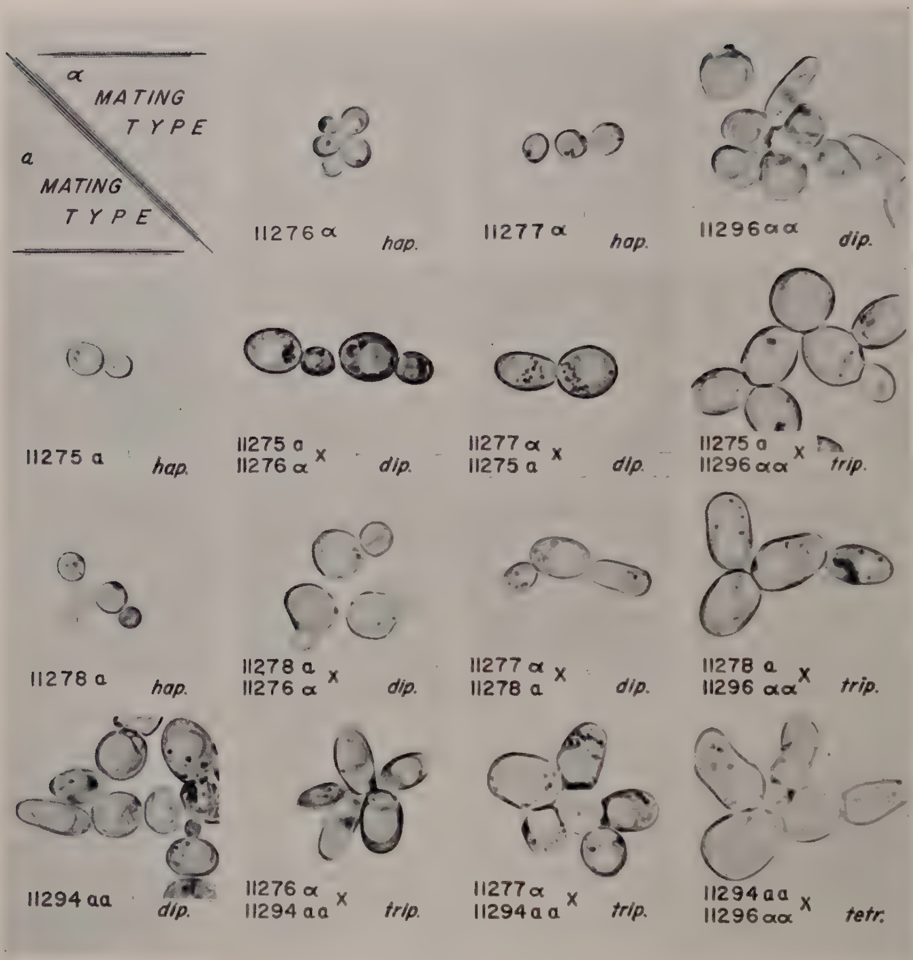
Culture 12370: *S G MA me MG W* spores

12390	*α*	*SU*	*G*	*MA*	*me*	*MG*	*P*	*ad*
12391	*a*	*su*	*g*	*MA*	*me*	*MG*	*P*	*ad*
12392	*α*	*su*	*G*	*MA*	*me*	*mg*	*W*	*AD*
12393	*a*	*SU*	*g*	*MA*	*me*	*mg*	*W*	*AD*
12401		*SU*	*G*	*ma*	*me*	*MG*		
12402		*SU*	*G*	*MA*	*me*	*mg*		
12403		*SU*	*g*	*MA*	*me*	*MG*		
12407		*su*	*G*	*ma*	*me*	*mg*		
12408		*su*	*g*	*ma*	*me*	*mg*		
12409		*SU*	*g*	*MA*	*me*	*MG*		
12410		*SU*	*G*	*MA*	*me*	*MG*		

Culture 12380: *S G MA ME MG W* spores

12411		*SU*	*g*	*MA*	*me*	*MG*	*W*
12412		*SU*	*g*	*MA*	*me*	*MG*	*W*
12413		*SU*	*g*	*MA*	*ME*	*MG*	*W*
12414		*SU*	*g*	*MA*	*ME*	*MG*	*W*
12436	*a*	*SU*	*G*	*MA*	*ME*	*mg*	*W*
12437	*α*	*SU*	*g*	*MA*	*ME*	*MG*	*W*
12438	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*
12439	*α*	*SU*	*g*	*MA*	*ME*	*MG*	*W*
12440	*a*	*su*	*G*	*MA*	*me*	*mg*	*W*
12441	*α*	*SU*	*g*	*MA*	*me*	*mg*	*P*
12442	*a*	*SU*	*G*	*MA*	*me*	*MG*	*P*
12443	*α*	*su*	*g*	*MA*	*me*	*MG*	*W*
12451	*α*	*SU*	*g*	*MA*	*ME*	*mg*	*P*
12452	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*
12453	*a*	*SU*	*g*	*MA*	*ME*	*MG*	*P*
12454	*α*	*SU*	*g*	*MA*	*ME*	*MG*	*W*
12455	*α*	*SU*	*g*	*MA*	*ME*	*MG*	*W*
12456				died			
12457	*a*	*SU*	*g*	*MA*	*ME*	*MG*	*W*
12458	*a*	*SU*	*G*	*MA*	*ME*	*mg*	*W*
12459	*α*	*SU*	*g*	*MA*	*ME*	*MG*	*W*
12460	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*
12461	*a*	*SU*	*g*	*MA*	*ME*	*mg*	*W*
12462	*α*	*SU*	*G*	*MA*	*ME*	*MG*	*W*

For explanation of symbols see Table 1.



C. C. Lindegren & Gertrude Lindegren—Tetraploid Saccharomyces. Plate 1

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EXPLANATION OF PLATE

Plate 1. A 'checkerboard' diagram with the gametes forming two outer rows. The *a* gametes form the column at the left and the α gametes form the top row. Each hybrid is located at the intersection of a row and a column. The tetraploid hybrid between the *aa* and the αα gametes is found in the lower right-hand corner. Four diploid hybrids, four triploid hybrids, and a single tetraploid hybrid were produced by matings between the six different gametes; four of the gametes are haploid and two are diploid. The haploid gametes are the smallest cells; the diploid gametes are considerably larger and are approximately the same size as the diploid hybrids. Matings between the haploid and diploid gametes produced four different triploid hybrids which are much larger than the diploid cultures. The single tetraploid produced by mating two diploid gametes is the largest hybrid. Photographs by Frank L. Kennedy, jun. Magnification × 3060.

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A Study of Chitin-decomposing Micro-organisms of Marine Origin

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SUMMARY: Twenty strains of aerobic, facultatively anaerobic, chitinoclastic bacteria have been isolated from marine mud by enrichment cultures. Each was able to derive its full carbon and nitrogen requirements from chitin. None was an obligate chitinovor.

These cultures comprised four new species of *Achromobacter*, two new species of *Pseudomonas*, one new species of *Flavobacterium* and one new species of *Micrococcus*. Detailed descriptions for each species are appended.

Each organism was able to liberate ammonia and reducing sugar from the chitin molecule. Glucosamine and acetic acid were not detected in the cultures, possibly because of their ready availability as supplementary nutrients.

Chitin is a tough leathery substance insoluble in the usual organic solvents, requiring strong mineral acid for its solution. It is a nitrogen-containing polysaccharide, generally believed to be composed of polymerized *N*-acetylglucosamine united by beta-1, 4 linkages to form long chains which are orientated in much the same manner as the glucose units in cellulose.

Chitin has a wide distribution in nature. It occurs in the exo-skeleton of crustacea, in some mollusca, coelenterata, protozoa (ZoBell, 1946) and in the cell wall of certain lower fungi and filamentous yeast (Nabel, 1939). Waksman (1933) has reported that it occurs as one of the nitrogenous constituents of marine sediments.

It has been estimated that several million tons of chitin are produced annually by the copepoda, a subclass of planktonic crustacea. This, as well as the chitin produced by other organisms in the sea, is probably utilized by biological agents because little accumulates in marine sediments, and, moreover, if the chitin were not decomposed its accumulation would soon cause a serious drain on the marine carbon and nitrogen (ZoBell & Rittenberg, 1938). There are few animals which can attack chitin, and it is generally agreed that its degradation in nature is largely due to microbial action.

Since Benecke (1905) reported the isolation of *Bacillus chitinovorus* from the polluted waters of Kiel harbour, there have appeared numerous reports of the isolation of micro-organisms capable of decomposing chitin. The reports of Steiner (1931), Johnson (1932), Waksman, Carey & Reuszer (1933), ZoBell & Anderson (1936), ZoBell & Rittenberg (1938), Hock (1941), Erikson (1941) and Stanier (1947) on the occurrence of chitin-decomposing micro-organisms from marine sources, together with the report of Stuart (1936) on the occurrence of halophilic chitinovorous bacteria in marine salt from various parts of the world, reveals the world-wide distribution of chitin-decomposing micro-organisms in the seas. Chitinovors have been isolated from terrestrial sources by Folpmers

(1921), Rammelberg (1931), Johnson (1932), Bucherer (1935), Benton (1935) and Skinner & Dravis (1937).

ZoBell & Rittenberg (1938), by the use of enrichment culture methods, isolated thirty-one different types of chitinoclasts and found that they differed greatly in their nitrogen and carbon (energy) requirements as well as their action on chitin. Chitinoclastic activity took place more readily aerobically than anaerobically. Many of their chitinoclasts produced acetic acid and ammonia, suggesting to them that certain chitinoclasts attack the chitin molecule by hydrolysis of the acetylated amino groups. Others were able to attack the essential carbon and oxygen linkages of the chitin molecule resulting in a complete disintegration and the liberation of reducing sugars.

Few of the previous reports on chitinoclastic bacteria have given enough information to justify an opinion as to whether the organisms isolated were representatives of known genera and species or not. The investigation here reported was undertaken to obtain some information on this point.

METHODS AND MATERIALS

Chitin was prepared from the exoskeleton of shrimp by the method of Benton (1935). The material was thoroughly washed in warm water and decalcified in cold 1 % (v/v) HCl, which was changed every day for 4 days. It was then washed, placed in 2 % (w/v) KOH for about 10 days, and on four separate occasions brought to a boil and allowed to cool. It was then washed free from alkali, cut in strips and extracted repeatedly with boiling ethanol and dried. The purified strips gave positive tests for chitin as listed by Dehn (1937), and by Glick (1949).

Enrichment cultures for chitin-decomposing micro-organisms were made by adding strips of purified chitin to a thin layer of dilute mineral base (K_2HPO_4 0·1 % (w/v), $MgSO_4$ 0·05 % (w/v), NaCl 0·05 % (w/v), $CaCl_2$ 0·01 % (w/v), $FePO_4$ trace) in flat glass bottles. A second series of enrichments was set up in the same manner, but with the addition of 0·1 % (w/v) NH_4Cl to the mineral base. The media were adjusted to pH 7·0, inoculated with marine mud and incubated at 25–30°.

For chitin agar plates the purified strips were dissolved in 50 % (v/v) H_2SO_4 and precipitated by rapid dilution with a 15-fold volume of water. The resulting finely divided chitin was freed from acid by washing on a Buchner funnel until the filtrate was neutral to litmus. This wet material was incorporated into the mineral base agar directly, as drying results in the formation of a hard mass which is impossible to resuspend.

Following serial enrichment cultures, streaks were made on the chitin agar plates. These were examined daily for the appearance of haloes, indicative of chitin decomposition, surrounding the colonies. Colonies showing haloes were isolated, purified and finally transplanted to nutrient agar slants for further study.

For studies on chitin decomposition, flat glass bottles containing thin layers of the dilute mineral base (pH 7·0) and strips of purified chitin were inoculated with pure cultures of the chitin-decomposing organisms. These were examined, at 2, 4, 6, 8, 12, 16 and 20 days, for ammonia, acetate, glucosamine, reducing sugars and for changes in pH. Changes in pH were measured electrometrically. Ammonia was determined with Nessler's reagent; glucosamine by the method of Elson & Morgan (1933), and reducing sugars with Barfoed's and Fehling's reagents.

The ability to use various carbon compounds was tested by adding 1 % (w/v) (unless otherwise noted) of the compound to the mineral base with 0·1 % (w/v) NH_4Cl, and observing for growth. Media for the study of other biochemical characters were prepared in the usual manner. The arrangement of the flagella of motile forms was determined by both Zettnow staining and electron microscope examination.

RESULTS

Chitin decomposition in enrichment cultures was manifested by erosion of the strips of chitin after 8–12 days at 25–30°. Gram-stained preparations from the enrichment cultures revealed the presence of a flora consisting of small rods and cocci. Streaks from enrichment cultures on chitin-mineral agar revealed, in 2–3 days, several colonial types surrounded by clear haloes in which the chitin had been dissolved.

Twenty colonies were picked and purified; from further study these appeared to include eight different species of chitin-decomposing organisms. All of the strains isolated were true bacteria and were distributed among the genera *Achromobacter*, *Pseudomonas*, *Flavobacterium* and *Micrococcus*. The failure to detect any of the myxobacteria types is probably due to the fact that they grow in an inconspicuous manner and at a slow rate, and thus may be easily overlooked (Stanier, 1947).

The eight species reported here have been studied in detail in regard to chitin decomposition, carbon utilization, and by various other morphological, biochemical and physiological tests. The positive results obtained from the study of chitin decomposition are summarized in Table 1. The results of the other tests are included as a part of the description of each organism. Species descriptions with the names which have been selected are appended to this report. All tests for acetate and glucosamine were negative. All cultures became acid after from 4 to 8 days. The pH range was from 5·5 to 6·0.

DISCUSSION

The organisms isolated in the present study were all representatives of well-known genera for which species descriptions seem not to have been recorded previously. Because of the incomplete descriptions of the aerobic bacteria with chitinoclastic ability which have been isolated by the few earlier workers who have studied such organisms it is not possible to identify any of the types here reported with previous isolates.

Probably the most extensive study of chitinoclastic bacteria of marine origin is that of ZoBell & Rittenberg (1938). Some of their isolates were able to grow on a medium consisting of purified chitin in sea water, others grew only if supplied with a supplementary source of carbon, and still others required that

Table 1. *Ammonia and reducing sugar from chitin*

	Day of observation													
	2		4		6		8		12		16		20	
Organism	NH_3	RS	NH_3	RS	NH_3	RS	NH_3	RS	NH_3	RS	NH_3	RS	NH_3	RS
Achr. labrum	All negative	All negative	All negative	All negative	++	All negative	++	+	++++	++	Not tested	++	Not tested	+++
Achr. ureasophorum					+		++	+	++++	++		+++		+++
Achr. lipophagum					++		++	–	++++	++		++		++
Achr. hyperopticum					++		++	–	++++	+		++		+++
Ps. cryothasia					++		+++	–	++++	+		+		+++
Ps. subrubra					–		++	–	++++	+		+++		+++
Fl. indoltheticum					+		+++	+	++++	+		+++		+++
M. colpogenes					++		++	++	++++	++		+++		+++

The intensity of the test is indicated by the number of +marks. Tests for reducing sugar ranged from a yellow (+) to an orange (+++). No brick red (++++) tests were obtained.

additional sources of both carbon and nitrogen be supplied. Despite the fact that chitin is presumably a polymerized glucosamine some of their cultures were unable to attack glucose, although able to effect the hydrolysis of starch. Some ZoBell & Rittenberg types grew without detectable effect on the chitin while others would dissolve it. They postulated that the chitin molecule is attacked either by hydrolysing the acetylated amino groups with the liberation first of acetic acid and then of ammonia, or by the hydrolysis of the carbon-nitrogen linkage with the liberation of acetamide from which ammonia and acetic acid were freed by further hydrolytic activity. They were able to detect acetic acid in some of their cultures. Most of their organisms were able to use the ammonium ion, but ammonia must have been produced in excess of the metabolic needs since apparently all cultures showed a positive test for ammonia. Not all types produced reducing sugar, and not all cultures developed acidity.

All organisms isolated in the present study were able to use ammonia as a source of nitrogen. All except one produced enough ammonia to show a positive test by the sixth day. One culture, *Pseudomonas subrubra*, was generally less vigorous than any of the others. It was not positive for ammonia until the eighth day, and for acid only by the twelfth day, although the other cultures were acid in from 4 to 8 days.

A positive test for acetate was not obtained with any culture at any time. If the theory of ZoBell & Rittenberg concerning the mechanism of chitin breakdown is correct, the failure to detect acetate may possibly be explained by the more ready availability and consequently preferential utilization of the acetate carbon to such an extent that it does not accumulate sufficiently in the culture to give a positive test. This theory finds support in the fact that maximum growth was more quickly attained in cultures where acetate was supplied as a supplementary source of carbon than where chitin was the sole carbon source.

The breakdown of the chitin molecule into glucosamine units is a possibility, but all tests for glucosamine were negative. Reducing sugar appeared in some cultures by the eighth day and in all by the twelfth day, although the positive reactions were not as intense as that of the glucose control. Since all cultures were able to utilize glucosamine it could be that this substance was preferentially metabolized as was suggested for acetate. All cultures produced acid from chitin in from 4 to 12 days.

Five of the eight types studied were proteolytic and four were lipolytic. All except two were weakly saccharolytic, producing acid without gas from the sugars which were attacked.

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CHARACTERISTICS OF ISOLATED STRAINS

Achromobacter labrum n.sp. (from the Greek, greedy)

Rods: 0·2–0·4 by 0·8–1·1μ, occuring singly, in pairs and occasionally in clumps. Motile by means of peritrichous flagella. Gram-negative.

Agar colonies: circular, smooth, entire, flat, glistening, opaque, non-pigmented to pale cream.

Agar slant: abundant filiform streak, smooth, glistening, opaque, non-pigmented.

Gelatin stab: no liquefaction.

Broth: moderate turbidity; surface growth adherent to the walls of the tubes.

Litmus milk: becomes acid in 6 days.

Hydrogen sulphide, indole and urease not formed.

Nitrites produced from nitrates.

Trimethylamine not produced from trimethylamine oxide, choline or betaine.

Gelatin, casein, and fats not hydrolysed; starch and chitin are hydrolysed.

Ammonia produced from peptone.

Produces acid but no gas from glucose, maltose, fructose, dextrin, mannose, trehalose, cellobiose. Acid not produced from lactose, sucrose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, dulcitol, inositol, melezitose, adonitol.

Carbon utilization: grows on glucose, lactose, sucrose, maltose, fructose, dextrin, mannose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, galactose, trehalose, inositol, cellobiose, melezitose, adonitol, chitin, glycogen, starch, butyrate, valerate, asparaginate, succinate, malate, fumarate (0·5 %), lactate, acetate, tartrate, citrate, pyruvate (0·3 %), levulinate, β-alanine, glucosamine, ethanol and *tert.*-butanol. No growth on dulcitol, cellulose, malonate, salicylate, oxalate, mandelate, benzoate (0·5 %), propionate, *n*-amyl alcohol, methanol, *n*-propanol, *iso*-amyl alcohol, lumichrome.

Growth not inhibited by 10 % sodium chloride.

Aerobic, facultative.

Optimum temperature: 20–30°. Grows at 4°.

Source: marine mud.

Habitat: sea water.

One additional strain.

Achromobacter ureasophorum n.sp. (from the Greek, urease producing)

Small rods: 0·2 by 0·8μ., occurring singly, motile by means of peritrichous flagella. Gram-negative.

Agar colonies: circular, raised, glistening, entire, translucent, non-pigmented.

Agar slant: abundant, filiform, glistening, translucent, non-pigmented.

Gelatin stab: no liquefaction.

Broth: slight turbidity, with a stringy sediment.

Litmus milk: becomes slightly acid in 6 days.

Hydrogen sulphide, indole, nitrites not produced.

Urease present.

Trimethylamine not produced from trimethylamine oxide, choline, or betaine.

Gelatin, casein and fats not hydrolysed; starch and chitin are hydrolysed.

Ammonia produced from peptone.

Produces acid but not gas from glucose, maltose, sucrose, fructose, mannose, trehalose, and cellobiose. Does not ferment lactose, dextrin, arabinose, rhamnose, xylose, raffinose, inulin, salicin, dulcitol, inositol, melezitose, adonitol.

Carbon utilization: utilizes glucose, lactose, sucrose, maltose, fructose, dextrin, mannose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, dulcitol, galactose, trehalose, inositol, cellobiose, melezitose, adonitol, glycogen, starch, chitin, asparaginate, succinate, malate, lactate, pyruvate (0·3 %), acetate, β-alanine, glucosamine, ethanol, methanol, and *tert.*-butanol. Does not utilize fumarate (0·5 %), malonate, tartrate, citrate, levulinate, propionate, salicylate, valerate, oxalate, butyrate, mandelate, benzoate (0·5 %), *n*-amyl alcohol, *iso*-amyl alcohol, *n*-propanol, lumichrome.

Growth not inhibited by 10 % sodium chloride.

Aerobic, facultative.

Optimum temperature: 20–30°. Grows at 4°.

Source: marine mud.

Habitat: sea water.

Achromobacter lipophagum n.sp. (from Greek, fat-eating)

Small rods: 0·4 by 1·0μ., occurring singly, in pairs, and small clumps, motile by means of peritrichous flagella. Gram-negative.

Agar colonies: circular, smooth, entire, glistening, opaque, and non-pigmented.

Agar slant: abundant, filiform, glistening, opaque and non-pigmented.

Gelatin stab: saccate liquefaction.

Broth: uniform turbidity.

Litmus milk: becomes acid in 4 days with slight peptonization.

Hydrogen sulphide, indole nitrites, urease not formed.

Trimethylamine produced from trimethylamine oxide but not from choline and betaine.

Gelatin, casein, fats, starch, and chitin are hydrolysed.

Ammonia produced from peptone.

Produces acid but not gas from glucose, maltose and mannose. Does not ferment lactose, sucrose, fructose, dextrin, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, sorbitol, salicin, dulcitol, trehalose, inositol, cellobiose, melezitose, adonitol.

Carbon utilization: utilizes glucose, lactose, sucrose, maltose, fructose, dextrin, mannose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, dulcitol, galactose, trehalose, inositol, cellobiose, melezitose, adonitol, glycogen, starch, chitin, asparaginate, succinate, malate, fumarate (0·5 %), lactate, pyruvate (0·3 %), acetate, β-alanine, glucosamine, ethanol, methanol, *tert.*-butanol. Does not utilize malonate, tartrate, citrate, levulinate, propionate, salicylate, valerate, oxalate, butyrate, mandelate, benzoate (0·5 %), *n*-amyl alcohol, *iso*-amyl alcohol, *n*-propanol, lumichrome.

Growth not inhibited by 10 % sodium chloride.

Aerobic, facultative.

Optimum temperature: 20–30°. Grows at 4°.

Source: marine mud.

Habitat: sea water.

Five additional strains.

Achromobacter hyperopticum n.sp. (from Greek, hard to please)

Small rods: 0·2 by 0·6μ., occurring singly, and in small clumps. Motile by means of peritrichous flagella, Gram-negative.

Agar colonies: small, punctiform, glistening, opaque and non-pigmented.

Agar slant: beaded, glistening, opaque and non-pigmented.

Gelatin stab: crateriform liquefaction.

Broth: moderate turbidity, surface ring adhering to the walls of the tube, and a slight stringy sediment.

Litmus milk: alkaline, with slight peptonization in 4 days.

Hydrogen sulphide, indole and urease not formed.

Nitrites are produced from nitrates.

Trimethylamine not produced from trimethylamine oxide, choline, or betaine.

Gelatin, casein, fats, starch and chitin are hydrolysed.

Ammonia produced from peptone.

Produces acid but not gas from glucose, maltose, mannose, trehalose and cellobiose. Does not ferment lactose, sucrose, fructose, galactose, arabinose, dextrin, rhamnose, xylose, raffinose, inulin, mannitol, inositol, melezitose, adonitol.

Carbon utilization: utilizes glucose, sucrose, dextrin, mannose, raffinose, galactose, cellobiose, starch, glycogen, chitin, glucosamine, acetate. Does not utilize succinate, malate, fumarate (0·5 %), lactate, malonate, tartrate, citrate, pyruvate (0·3 %), levulinate, β-alanine, asparaginate, propionate, salicylate, valerate, oxalate, butyrate, mandelate, benzoate (0·5 %), ethanol, methanol, *iso*-amyl alcohol, *n*-amyl alcohol, *tert.*-butanol, *n*-propanol, lumichrome.

Growth not inhibited by 10 % sodium chloride.

Aerobic, facultative.

Optimum temperature: 20–30°. Grows at 4°.

Source: marine mud.

Habitat: sea water.

One additional strain.

Pseudomonas cryothasia n.sp (from Greek, cold enduring)

Small rods: 0·3–0·5 by 0·8–1·2μ., occurring singly, in pairs and small clumps. Motile by means of monotrichous flagellum.

Gram-negative.

Agar colonies: circular, smooth, entire, convex, opaque, non-pigmented to pale cream.

Agar slant: filiform, glistening, opaque, non-pigmented to pale cream.

Gelatin stab: saccate to infundibuliform liquefaction.

Broth: moderate turbidity, slight surface scum, and slight flocculent sediment.

Litmus milk: acid with peptonization in 6 days.

Hydrogen sulphide, indole and urease not formed.

Nitrites are produced from nitrates.

Trimethylamine not produced from trimethylamine oxide, choline, or betaine.

Gelatin, casein, fats and chitin are hydrolysed; starch is not hydrolysed.

Acid or gas not produced from glucose, lactose, sucrose, maltose, fructose, dextrin, mannose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, dulcitol, galactose, trehalose, inositol, cellobiose, melezitose, adonitol.

Carbon utilization: utilizes glucose, maltose, dextrin, glycogen, pyruvate (0·3 %), acetate, asparaginate, succinate, malate, lactate, citrate, chitin, glucosamine. Does not utilize lactose, sucrose, fructose, mannose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, dulcitol, galactose, trehalose, inositol, cellobiose, melezitose, adonitol, fumarate (0·5 %), malonate, tartrate, levulinate, β-alanine, propionate, salicylate, valerate, oxalate, butyrate, mandelate, benzoate (0·5 %), starch, methanol, ethanol, *n*-amyl alcohol, *iso*-amyl alcohol, *tert.*-butanol, *n*-propanol, lumichrome.

Growth not inhibited by 10 % sodium chloride.

Aerobic, facultative.

Optimum temperature: 20–30°. Grows at 4°.

Source: marine mud.

Habitat: sea water.

Two additional strains.

Pseudomonas subrubra n.sp. (from Latin, reddish)

Rods: 0·5–0·7 by 1·0–2·2 μ., occurring singly, and in pairs. Motile with a single polar flagellum. Gram-negative.

Agar colonies: circular, smooth, entire, glistening, opaque, non-pigmented to pale pink.

Agar slant: filiform, smooth, glistening, opaque, non-pigmented to pale pink streak.

Gelatin stab: crateriform liquefaction.

Broth: moderate turbidity, fine granular sediment.

Litmus milk: acid with peptonization in 72 hr.

Hydrogen sulphide, indole and urease not formed.

Nitrites produced from nitrates.

Trimethylamine not produced from trimethylamine oxide, choline, or betaine.

Gelatin, casein, fats starch and chitin are hydrolysed.

Ammonia produced from peptone.

Produces acid but no gas in glucose, maltose, fructose, dextrin, mannose, trehalose and cellobiose. Acid not produced in lactose, sucrose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, dulcitol, inositol, melezitose, adonitol.

Carbon utilization: growth on glucose, sucrose, maltose, fructose, dextrin, mannose, rhamnose, mannitol, galactose, trehalose, inositol, cellobiose, chitin, glycogen, starch, butyrate, valerate, asparaginate, glycine, succinate, malate, fumarate (0·5 %), lactate, acetate, citrate, pyruvate (0·3%), levulinate, β-alanine, glucosamine, ethanol, methanol, *tert.*-butanol. No growth on lactose, arabinose, xylose, raffinose, inulin, salicin, dulcitol, melezitose, adonitol, malonate, tartrate, propionate, salicylate, oxalate, mandelate, benzoate (0·5 %), *n*-amyl alcohol, *iso*-amyl alcohol, *n*-propanol and lumichrome.

Growth not inhibited by 10 % sodium chloride.

Aerobic, facultative.

Optimum temperature: 20–30°. Grows at 4°.

Source: marine mud.

Habitat: sea water.

One additional strain.

Flavobacterium indoltheticum n.sp. (from Greek, indole making)

Rods: 0·4–0·9 by 1·0–1·5μ., occurring singly, in pairs and occasional clumps. Motile by means of peritrichous flagella. Gram-negative.

Agar colonies: circular, smooth, entire, opaque, yellow to yellow-orange pigment.

Agar slant: yellow to yellow-orange streak, smooth, opaque.

Gelatin stab: crateriform liquefaction.

Broth: turbid, slight surface scum, yellow to orange sediment.

Litmus milk: acid in 48 hr.

Hydrogen sulphide, nitrites and urease not formed.

Indole is formed.

Trimethylamine not produced from trimethylamine oxide, choline or betaine.

Gelatin, casein, starch and chitin are hydrolysed; fats not hydrolysed.

Ammonia produced from peptone.

Acid but not gas produced in glucose, sucrose, maltose, fructose, dextrin, mannose, raffinose, trehalose and cellobiose. Acid not produced in lactose, arabinose, rhamnose, xylose, inulin, mannitol, salicin, dulcitol, inositol, melezitose, adonitol.

Carbon utilization: growth on glucose, sucrose, maltose, fructose, dextrin, mannose, raffinose, salicin, cellobiose, trehalose, pyruvate (0·3 %), acetate, chitin, starch, glycogen and glucosamine. No growth on lactose, arabinose, rhamnose, xylose, inulin, mannitol, dulcitol, galactose, inositol, melezitose, adonitol, succinate, malate, fumarate (0·5 %), lactate, malonate, tartrate, citrate, levulinate, β-alanine, asparaginate, propionate, salicylate, valerate, oxalate, butyrate, mandelate, benzoate (0·5 %), ethanol, methanol, *n*-amyl alcohol, *iso*-amyl alcohol, *tert.*-butanol, *n*-propanol, lumichrome.

Growth is inhibited by 10 % sodium chloride.
Aerobic, facultative.
Optimum temperature: 20–30°. Does not grow at 4°.
Source: marine mud.
Habitat: sea water.
One additional strain.

Micrococcus colpogenes n.sp. (from Greek, gulf-born)
Cocci, occurring in clumps and occasional pairs. Non-motile, Gram-positive.
Agar colonies: circular, smooth, entire, raised, glistening, yellow.
Agar slant: filiform, glistening, yellow streak.
Gelatin stab: no liquefaction.
Broth: moderate turbidity, with slight granular sediment.
Litmus milk: unchanged.
Hydrogen sulphide and indole not formed.
Nitrites and urease are formed.
Trimethylamine not produced from trimethylamine oxide, choline, or betaine.
Gelatin, casein, fats and starch are not hydrolysed; chitin is hydrolysed.
Acid or gas not produced from glucose, lactose, sucrose, maltose, fructose, dextrin, mannose, arabinose, rhamnose, xylose, raffinose, inulin, mannitol, salicin, dulcitol, galactose, trehalose, inositol, cellobiose, melezitose, adonitol.
Carbon utilization: utilizes glucose, maltose, mannitol, malate, pyruvate (0·3 %), acetate, succinate, glycogen, chitin, glucosamine.
Does not utilize fumarate (0·5 %), lactate, malonate, tartrate, citrate, levulinate, β-alanine, asparaginate, propionate, salicylate, valerate, oxalate, butyrate, mandelate, benzoate (0·5 %), starch, ethanol, methanol, *n*-amyl alcohol, *iso*-amyl alcohol, *tert.*-butanol, *n*-propanol, lumichrome.
Growth is inhibited by 10 % sodium chloride but not by 4 %.
Aerobic, facultative.
Optimum temperature: 20–30°. Does not grow at 4°.
Source: marine mud.
Habitat: sea water.
One additional strain.

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The Effect of Amino-Acids and Other Substances on the Activity of Streptococcal Hyaluronidase

With a Note on the Activity of Fractions obtained during the Isolation and Purification of Components of Streptococcal Hyaluronidase

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SUMMARY. The titre of streptococcal polysaccharidase was increased by incubation with β-alanine, serine, tyrosine, cystine, cysteine or methionine. Dextrinogenic amylase and hyaluronidase measured by a turbidity end-point were both activated by cysteine, cystine, serine or methionine. Polysaccharidase activity also increased after incubation with hyaluronic acid, starch, pectin or dextran. Pantothenic acid suppressed activity of SH-activated enzymes, and the rise of titre observed during incubation with substrate was decreased twofold or fourfold by addition of valine. The activation of streptococcal proteinase by cysteine was also prevented or retarded by pantothenic acid or valine. Pantothenyl alcohol was more active than either pantothenate (DL-calcium salt) or DL-valine.

Non-specific depolymerizing activity by hyaluronidase and amylase of Group A streptococci was reported by Crowley (1951), who also found that pro-enzyme in inactive cultures was activated by thiol groups, partially activated by metallic ions, and that pantothenic acid caused a transient rise of titre of inactive cell free filtrates. Hyaluronidase was decreased twofold in the presence of high proteinase concentration but there was no evidence to explain the proteinase effect. The present paper reports experiments made with partially purified polysaccharidase, with reference to amino-acids and other substances which activate polysaccharidase and proteinase.

MATERIAL AND METHODS

Strains. The strains of Group A streptococci were described in previous papers (Crowley, 1950, 1951). The serological classification and polysaccharidase activity are shown in Table 1.

Enzymic material was prepared from cell free filtrates of 2-day broth cultures. A note on the method of isolation and the activity of fractions obtained during purification is appended to this paper.

Activity. Amylase. Hydrolysis of 0·2 % soluble starch was tested by the method of Crowley (1951). *Proteinase.* The clotting time of milk + thiolacetate was tested by the method of Elliott & Dole (1947).

Hyaluronidase was tested by the M.C.P. method of McClean (1942), but human synovial fluid was used as substrate. Synovial fluid was obtained from knee joints at autopsy by first injecting 2 ml. sodium β-glycerophosphate (0·126 M) and withdrawing mucin + buffer which was collected in a sterile bottle and immediately layered over with toluene. Volumes of fluid obtained varied from 3 to 5 ml., therefore all samples were pooled. The first precipitate which developed while standing under toluene, together with cell debris, was removed by centrifuging. The 'clean' pool was maintained at a volume not less than 150 ml., and 25–30 ml. removed at any one time to make the test substrate. Standing under toluene at −4° or at bench temperature the viscosity remained very constant, and the pH value was in the range 6·8–7·2. A sample from the original pool contained 1 % hyaluronic acid (this estimation was kindly made by Dr W. T. Morgan). A dilution of synovial fluid in distilled water containing approximately 0·2 % hyaluronic acid clotted with 2N acetic acid without additional protein. The hyaluronidase titre of a Type 4 strain (H713) was estimated using a cell free filtrate which was concentrated by dialysis against glycerol and stored at −10°. Subsequently, this 'standard' enzyme was used to estimate the strength of fresh batches of substrate. Usually 1 vol. of synovial fluid was diluted with 5 vol. of distilled water, and diluted by further half volumes as required. Enzyme samples in buffer + saline (Na β-glycerophosphate 0·063M + NaCl 0·1M) were diluted in distilled water at 20 % differences. The M.C.P. test was carried out at pH 7·0.

Method of reading results. The M.C.P. end-point was recorded in the usual way, but a record was also made of the greatest dilution of enzyme which was associated with an *increased* turbidity on acidification compared with two control tubes containing substrate + water or + buffer saline. (This end-point differs from the end-point observed in the usual turbidity reduction tests.) After standing overnight at bench temperature further readings were taken on lines similar to the ACRA test (Burnet, 1948). Coarse flocculation was seen in dilutions sometimes × 4 higher than the M.C.P. end-point, and increased turbidity was seen just above as well as several tubes below the M.C.P. end-point in some conditions. Meyer (1947), using testicular hyaluronidase, has suggested that the M.C.P. and turbidimetric methods may not measure the same enzyme. After streptococcal hyaluronidase had been considerably purified turbidity changes were seen in synovial mucin substrate which were not apparent using purified umbilical hyaluronate + horse serum, or + crystalline albumin (horse or human). In this connexion changes in starch, pectin and dextran substrates were also best observed with purified enzymes, and moreover differences between enzymes from different strains became clearer with increasing purification. It was convenient to designate the primary depolymerizing activity as the β-activity or liquefying component, and the turbidity changes as α-activity (see p. 916). It was not clear whether two enzymes or two aspects of the same enzyme were measured.

Units. The end-point tube contained one unit of enzyme in 0·5 ml.; therefore, 1 ml. of undiluted samples contained 2 × reciprocal of endpoint dilution.

Polysaccharide substrates. Dextran (*Leuconostoc mesenteroides*) was supplied in 6 % solution prepared for intravenous use by the East Anglia Chemical Co. The solution was boiled for 60 min. before use. Citrus pectin (Messrs Reckitt and Colman Ltd., Carrow Works, Norwich) was twice precipitated from aqueous solution with 1·5 vol. of pure acetone. Five samples of umbilical hyaluronate were used at different times, of which three were commercially prepared. Crystalline albumin was given by Dr R. A. Kekwick.

EXPERIMENTAL

The effect of pantothenic acid and amino-acids on activity of hyaluronidase isolated from cell free filtrates

Enzymic material was prepared from culture filtrates of ten strains of Group A streptococci (Table 1).

Table 1. *Strains of Group A streptococci used as source of extracellular enzymes*

Group A streptococci. Strains	Serological type	Polysaccharidase activity		
		Hyaluronidase	Amylase	Depolymerase
BB	Type 4 (M)	++	++	++
H713	Type 4 (M)	++	++	++
765/E	T antigen group 4, 24, 26, 28, 29, 46	++	++	++
P315		+	±	+
R1823	Type 24 (M)	−	+	+
6794	Type 28 (M)	−	+	+
63T	Type 22 (T)	++	−	+
P674	Type 22 (T)	++	−	+
4226	Type 2 (M)	−	++	+
1182	Untypable	−	+	+

(M, T = antigen identified; ++, +, ± = degrees of activity; −, enzyme component absent or inactive.)

Enzyme samples in buffer + saline were mixed with equal volumes of polysaccharide substrate (0·2 % aqueous solution), namely hyaluronate, starch, pectin and dextran. Enzyme was also distributed in samples (*a*) without substrate, (*b*) with test substance before addition of substrate, (*c*) with test substance after preliminary incubation with substrate. Test samples were removed at hourly or daily intervals during incubation at 37° and frozen until the end of the experiment. Changes in enzyme titre were measured in terms of hyaluronidase or amylase. Pantothenic acid (DL-calcium salt), pantothenyl alcohol, or DL-amino-acids were added in 0·002 M concentration in the first instance, but tests were also made with 0·004, 0·02 and 0·04 M concentrations.

The effect of incubation with substrate. Hyaluronidase titre increased after incubation with starch, pectin or dextran for 2–4 days. Amylase titre of enzymes from three hyaluronidase positive strains showed equivalent increase during incubation with hyaluronate, pectin and dextran. Enzyme from each strain showed greatest activity with one particular substrate suggesting some specificity (Table 2) which agreed with the previous estimations of predominant activity (Table 1). No bacterial growth occurred during incubation. These results suggested either that an inactive precursor was present in the partially purified material or that inactivated enzyme protein was reactivated. The possibility of adaptation in the sense that substrate protected enzyme from inactivation during incubation had also to be considered.

The effect of pantothenic acid and β-alanine. Fig. 1 shows the hyaluronidase titre of enzyme from a Type 4 strain (P315) during incubation with pantothenyl alcohol. Similar results were recorded for five enzymes from hyaluroni-

Table 2. *Increased hyaluronidase activity after incubation with four substrates: relative specificity of components*

Purified enzyme from filtrate	Relative rise of titre after 4 days, 37° with substrates (0·2 % aq. dilution)			
Strain	Hyaluronic acid	Starch	Pectin	Dextran
H713	+++	+++	+	+
765/E	+++	+++	+	+
BB	++	++	+	+
P315	+++	+	+	+
R1823	+	++	+	+
6794	+	++	+	+/−
63T	+++	+	+++	+
P674	+++	+	++	+
4226	+	++++	+++	+/−
1182	+	++	+	+

(+ = twofold rise of titre, each additional + = × 2 increase; +/− = less than × 2 increase after 4 days.)

Enzymes were diluted in buffer + saline. M.C.P. and starch hydrolysis tests were made on samples taken at daily intervals. The final estimations were made after dialysis of enzyme + substrate mixture against water and buffer + saline to remove the products of the reaction.

dase positive strains. By comparison with control solutions the turbidity titre was decreased in the presence of pantothenic acid. While this effect was maintained by pantothenyl alcohol during prolonged incubation (10 days at 37°), pantothenate was less active. The results of incubation with β-alanine are shown in Fig. 2, using another Type 4 enzyme (BB). Amylase activity was used as a screening test comparing enzymes from ten strains. Pantothenic acid also suppressed dextrinogenic amylase; starch solution, however, showed loss of viscosity and flocculation though remaining blue with iodine. When hyaluronic acid, starch, pectin or dextran were added during incubation of enzyme with pantothenic acid, the rise of hyaluronidase or amylase titre was greater than with substrate alone. Only the β-activities were increased immediately; α-activities were suppressed for as long as 10 days in the presence of pantothenyl alcohol.

The effect of DL-*cysteine*. Figs. 1 and 2 also show the results of incubation with cysteine on the hyaluronidase activity of the same enzymes. Cysteine activated hyaluronidase, the turbidity titre showing relatively greater increase than the M.C.P. titre. Amylase was also activated, the rise of titre following the pattern of the turbidity curve. Ravel & Shive (1946) reported that biological antagonism between pantothenic acid and cysteic acid was neutralized by β-alanine.

The effect of DL-*amino-acids*. Thoai, Roche & Roger (1947) reported the reactivation of inactive alkaline phosphatase by alanine. Edlbacher & Wiss (1946) reported that histidine was the most efficient activator of D-amino oxidase. The effect of certain DL-amino-acids was tested using a Type 4 and a Type 22 enzyme. Hyaluronidase titre increased after incubation with serine, tyrosine or cystine, but neither alanine nor histidine affected activity.

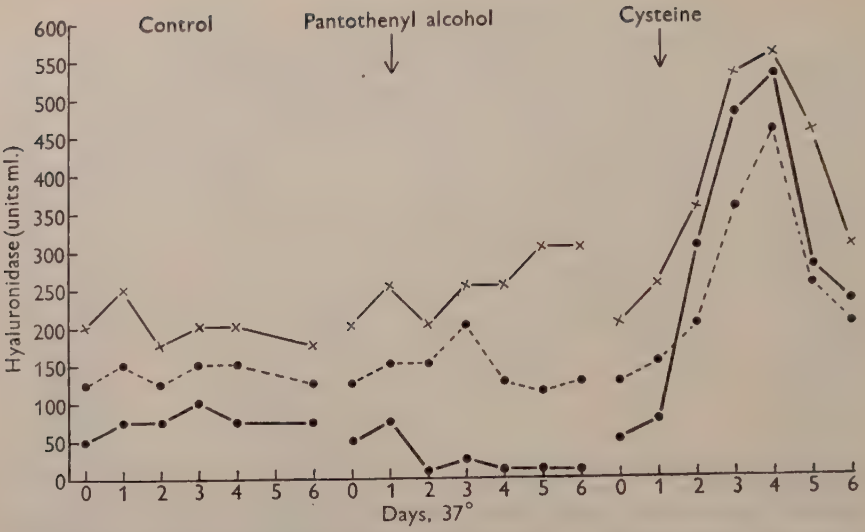

Fig. 1. Activity of streptococcal hyaluronidase during incubation with pantothenyl alcohol and cysteine. ----, M.C.P. titre; ×——×, primary depolymerase (β-activity); ●——●, increased turbidity of synovial mucin (α-activity). Purified enzyme from Type 4 streptococcus (P315) incubated for 7 days at 37° in buffer + saline, pantothenyl alcohol (0·04 % v/v) or cysteine (0·002 M) added after 1 day.

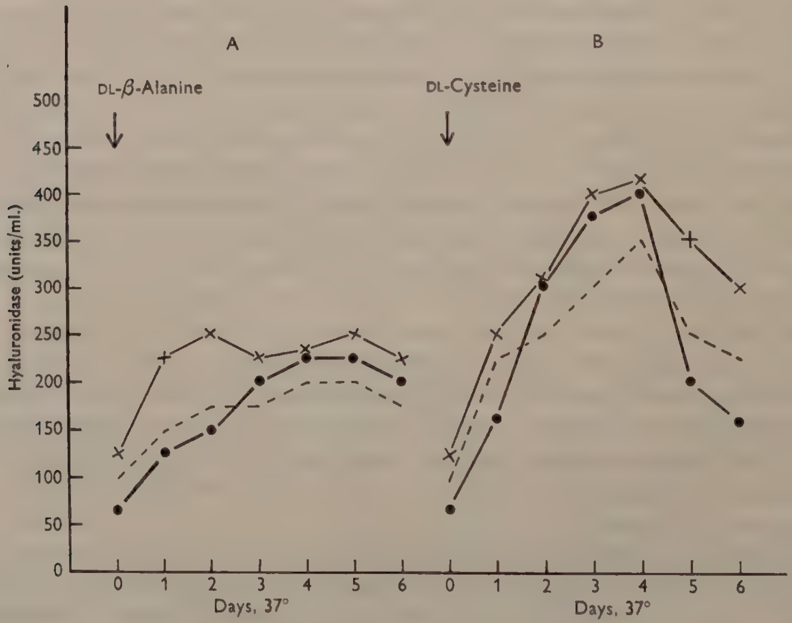

Fig. 2. The effect of β-alanine on hyaluronidase activity compared with that of cysteine. ----, M.C.P. titre; ×——×, depolymerase; ●——●, turbidity. Amino-acids (0·002 M) added to enzyme from Type 4 strain BB. Experimental conditions as in Fig. 1.

Table 3. *The activation of purified streptococcal hyaluronidase by* DL-*amino-acids; the effect on enzymes isolated from six strains*

DL-Amino-acids	Concentration (M)	Effect on titre of hyaluronidase components: Liquefying-depolymerizing activity Stages I and II	Effect on titre of hyaluronidase components: Increased turbidity of depolymerized substrate Stage III
Alanine	0·02	–	–
β-Alanine	0·02	+	+/–
Serine	0·002	++	++
Tyrosine	0·02	+	+/–
Histidine	0·02	–	–
Cysteine	0·002	.	+++
Cystine	0·002	.	+++
Methionine	0·002	.	+++
Valine	0·02	+/–	Decrease
Arginine	0·02	–	–
Proline	0·02	+/–	Decrease

(+/– = variable; – = no change; + = twofold rise of titre, each additional + sign = further twofold rise.)

The results of tests with a number of DL-amino-acids are shown in Table 3. With the exception of methionine, only certain alanine derivatives promoted polysaccharidase activity, but valine and proline decreased hyaluronidase titre of four different enzymes. Threonine, glycine and hydroxyproline reduced the titre of one enzyme but this finding has not been confirmed. At present, constituents of proteins found in animal tissues (Rose, Haines & Johnson, 1942) and bacterial phosphoprotein have been tested.

The effect of DL-*valine.* Twofold or fourfold reduction of hyaluronidase titre occurred after addition of valine (Fig. 3). The effect was neutralized by equimolar concentrations of cysteine, cystine or methionine, which also neutralized the effect of pantothenic acid. After a few hours the reaction was spontaneously reversed. The required concentration of thiol or potential thiol groups to reverse the effect of either pantothenic acid or valine varied for enzymes from different strains. The action of methionine was more prolonged than equivalent concentration of cysteine or cystine. The rise of turbidity titre coincided with a fall of pH value to 6·2–6·4, and with a fall of OR potential (Methylene blue indicator). In systems containing pantothenic acid, spontaneous reversal of the effect of thiol amino-acids coincided with re-oxidation of methylene blue.

Anti-proteinase activity. Proteinase was separated from polysaccharidase by precipitation with 25 % ethanol at pH 5·6. Purified polysaccharidase showed no proteinase activity, and two of the ten strains were proteinase negative. After cysteine was used to activate hyaluronidase or amylase, proteinase was detected in some of the purified enzymes, including the two preparations from proteinase negative strains. This suggested that the material was contaminated with inactive proteinase precursor (Elliott & Dole, 1947) and it was used to study the proteinase effect on hyaluronidase activity. It was found that high concentration of proteinase in culture filtrates was correlated with twofold reduction of hyaluronidase titre (Crowley, 1951). The inhibiting concentration was constant for the strain but showed considerable strain variation. There was no evidence to explain the effect.

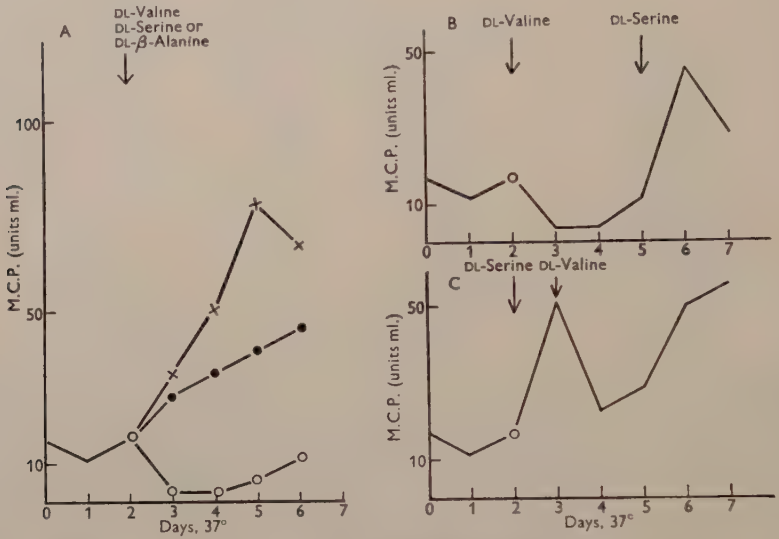

Fig. 3. Decrease of M.C.P. after addition of valine, compared with the effect of β-alanine or serine. A, M.C.P. titre before addition of amino-acids, M.C.P. titre after addition of valine, ○——○; β-alanine, ●——●; serine, ×——× (0·002 M). B, reversal of valine effect by serine. C, reversal of serine effect by valine. Purified enzyme from Type 4 strain (H713). Experimental conditions as for Figs. 1 and 2.

The concentration of proteinase in purified polysaccharidases varied widely between different preparations. The appearance of proteinase coincided with a fall of pH value to pH 6·0 or lower, and increasing proteinase activity was associated with decreasing hyaluronidase activity.

Adjustment of the pH to 7·4 with Na glycerophosphate partially neutralized the effect of increasing proteinase activity. The addition of hyaluronic acid also delayed the usual fall of hyaluronidase titre. These results suggested that proteinase inactivated but did not destroy hyaluronidase.

Not unexpectedly the activation of precursor by cysteine was prevented by pantothenic acid and by valine in the experimental conditions described. Pantothenyl alcohol suppressed proteinase activity indefinitely when the autocatalytic conversion of precursor would normally be expected. This result may perhaps be explained by a change in OR potential in the presence of pantothenic acid. Using methylene blue as a rough indicator 'turbidity' hyaluronidase and dextrinogenic amylase were activated at a higher OR potential than proteinase.

The effect of amino-acids on material isolated from inactive cultures

Hyaluronidase activity was reduced in cultures grown at 23° in filtered broth, and in broth containing proteose peptone or bicarbonate buffer (Crowley, 1951). Inactive cell-free filtrates were treated with $(NH_4)_2SO_4$ to give 25, 50 and 75 % saturation. Weakly active material was precipitated at all concentrations. This material was incubated with test amino-acids in the same way as the active enzymes.

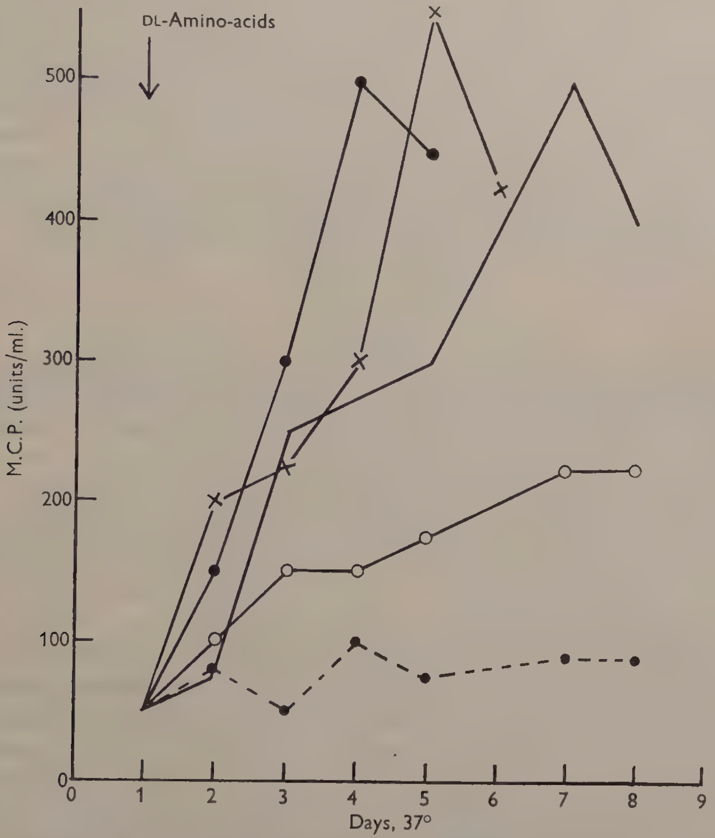

Fig. 4. The M.C.P. titre of material prepared from inactive culture after incubation with amino acids. M.C.P. titre of control, – – – –; M.C.P. titre with ●——●, serine; ×——×, methionine; ————, cysteine; ○——○, β-alanine. Amino-acids in 0·002 M concentration, added to solution of fraction precipitated by $(NH_4)_2SO_4$, 0·5 sat., from inactive culture filtrate. Two-day culture of Type 4 strain BB, grown at 23° proteose peptone filtered broth. The volume of solute was approximately one-tenth of the original volume of filtrate.

The effect of amino-acids. Fig. 4 shows the results of M.C.P. tests on material from inactive cultures of a Type 4 strain (BB) before and after incubation with β-alanine, serine, cysteine and methionine.

The rise of hyaluronidase titre followed the pattern usually seen when this strain was grown at 37° in culture medium favourable for hyaluronidase production.

The final yield of active enzyme was greatest from cultures grown at 23° in proteose peptone filtered broth buffered with sodium β-glycerophosphate. This medium also contained the greatest number of viable organisms. Two- or three-day cultures were treated with 3 vol. saturated $(NH_4)_2SO_4$ solution. An aqueous suspension of the precipitate was treated with a 75–80 % saturated solution of $(NH_4)_2SO_4$, resuspended and twice reprecipitated. After dialysis against running water until the dialysate gave no colour with Nessler reagent, and against buffer + saline, no viable organisms were detected. Any insoluble material was removed by filtration through paper, and the solution was filtered through a Seitz pad. After incubation with serine + methionine, hyaluronidase titre showed 12-fold increase. It seemed possible that products in the immediate environment of the organism, or intracellular constituents, provided a rich source of pro-enzymic material.

DISCUSSION

The possibility of an inactive hyaluronidase precursor analogous to streptococcal proteinase precursor (Elliott & Dole, 1947) was previously postulated on the basis of the similarity of environmental conditions affecting production of both enzymes (Crowley, 1951). The experiments described suggest that a component of a complex hyaluronidase or polysaccharidase may be derived from an inactive pro-enzyme and be activated by SH groups at a higher pH value and OR potential than proteinase. There is no evidence that the increased turbidity of synovial fluid substrate seen with purified streptococcal enzymes + potential thiol groups was in fact due to a hyaluronidase. All the material was contaminated with proteinase precursor, and the possibility of an artefact seems worth consideration. It might be possible that in some circumstances proteinase could attack the protein moiety of a protein-polysaccharide complex. In this connexion increased turbidity of synovial mucin has frequently been seen with crude culture filtrates of Group A streptococci of various serological types when hyaluronidase activity could not be demonstrated by any other method (unpublished data). These strains had one feature in common, namely, proteinase activity.

As a third possibility the amino-acids may have reversed inhibitions by factors at present unknown. Meyer & Rapport (1951) have shown that SH compounds including cysteine reversed the inhibition of testicular hyaluronidase by heavy metals. Weil-Malherbe (1948) suggests that apparent activation of enzymes by amino-acids is a non-specific protective affect against the hazards of purification *in vitro*. The inactive material prepared from cultures and cell free filtrates which subsequently yielded active enzymes did not merit the description 'purified'. The same groups of amino-acids appeared to activate hypothetical components in both crude and considerably purified material. There is insufficient evidence at present to support a theory of specific activity by amino-acids or by the pantoyl residue in relation to these streptococcal enzymes.

I wish to thank Dr Alexander Lawson and Dr J. H. Humphrey who gave me valuable help and advice.

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A NOTE ON THE ACTIVITY OF FRACTIONS OBTAINED DURING THE ISOLATION AND PURIFICATION OF COMPONENTS OF STREPTOCOCCAL HYALURONIDASE

The enzymes used above were isolated during attempts to purify and separate components of streptococcal polysaccharidase. Finally there was insufficient material for the usual criteria of purity to be applied.

Isolation of material

Filtrates of 2-day broth cultures (37°) were treated with $(NH_4)_2SO_4$ to bring to 70 % saturation, and left standing at bench temperature for 3 days. The precipitate was suspended in distilled water, filtered through paper, and the paper washed with phosphate buffer (pH 7·0). The solution was treated with $(NH_4)_2SO_4$ at 25, 50 and 75 % saturation successively (Table 1). The bulk of active material was precipitated at 50 % saturation, and this fraction contained streptococcal proteinase. The fraction was dissolved in water and reprecipitated by $(NH_4)_2SO_4$ at a lower pH value, when better separation was obtained. No progress was made until two types of polysaccharidase activity were distinguished: (1) depolymerizing activity, and (2) dextrinogenic amylase activity, which was associated with hyaluronidase measured by turbidity, as described on p. 907 of this *Journal*. These activities were designated β and α and further purification was attempted following the method of Wittler & Pillemer (1949) using methanol or ethanol at varied concentration and pH value in the cold.

Separation of polysaccharidase components by solubility in alcohol + water mixtures

The material isolated by salting out with $(NH_4)_2SO_4$ was dialysed against running water until the dialysate was Nessler negative, and against phosphate buffer until the bulk of material was in solution. The protein concentration of the soluble material from ten strains varied between 10·9 and 18·9 %. The results of fractional precipitation by methanol or ethanol are shown in Tables 2 and 3. Fraction I showed dextrinogenic amylase and 'turbidity' hyaluronidase activity. Precipitates were formed with 26 % Na_2SO_4 (at 37°), with 50 % $ZnSO_4$ and 3 % sulphosalicylic acid. The protein-copper test was positive, and the material was precipitated from solution by dialysis against water. Non-specific depolymerase was associated with Fraction II, which showed activity against hyaluronic acid, starch, pectin and dextran as substrates. Deoxyribonuclease tested by the method of McCarty (1948) was present in Fraction II of all ten strains. Precipitates formed with tannic acid, trichloroacetic acid and lead salts, but not with 26 % Na_2SO_4 at 37°. The protein-copper test was negative. After addition of cysteine, streptococcal proteinase was detected in this fraction, suggesting that inactive precursor was present. Elliott (1950) reported that inactive precursor was

Table 1. *Activity of polysaccharidase components obtained during isolation and purification with* $(NH_4)_2SO_4$

(Precipitated at bench temperature, pH value adjusted by $Na_2HPO_4 + NaH_2PO_4$ (M/15 conc.). Precipitates dissolved in distilled water.)

$(NH_4)_2SO_4$ (% saturation)	pH	Amylase	Hyaluronidase	Proteinase
25	7·0	+	+	+
50	7·0	++++	++++	++++
75	7·0	+/−	++	+/−
50 % fraction, $(NH_4)_2SO_4$				
50	6·0	++	++	+
.	6·4	++++	++++	+
70	7·0	+/−	++	−
>70	7·0	+/−	+	−

50 % pH 6·4 fraction		Polysaccharidase activity	
$(NH_4)_2SO_4$ (%)	pH value	Components activated by thiol groups α	Depolymerases liquefying components β
50	6·0–6·4	++++	+
70	7·0	+	+++
>70	7·0–7·4	+	++

++++ to +/− = relative activity.

Table 2. *Solubility of polysaccharidase components in methanol or ethanol at various pH values*

(Material was precipitated at −10°; precipitates were dissolved in phosphate buffer. Protein concentration in solutions 3–5 %.)

Methanol (%)	pH value	Polysaccharidase activity	
		β	α
25	6·4	++	++++
40	6·0	++++	+
	5·6	+	+
	4·8	+	+
	4·2	+	+
70	5·6	+	+
	7·0	++	+

Ethanol (%)	pH value	Polysaccharidase activity	
		β	α
18	5·2	+	+++
25	6·4	+	++++
40	5·6	+	+/−
	4·8	++++	+/−
	4·2	+++	+/−
70	5·6	++	+
	6·8	++	+

+ = activity based on unit/mg. PN_2, each additional + = twofold increase; +/− = weak activity.

Table 3. *Activity of polysaccharidase components after further purification with methanol + water mixtures*

Fraction I, 25 % methanol, pH 6·4		Polysaccharidase components	
Methanol (%)	pH value	α	β
25	6·4	++++	+
40	6·0–4·8	No precipitate	
50	4·2	Faint precipitate	
>50 + $CaCl_2$, 0·0025 M	4·2–6·8	+/−	++

Fraction II, 40 % methanol, pH 6·0		Polysaccharidase components	
Methanol (%)	pH value	α	β
25	6·4	No precipitate	
40	4·8	+/−	++++
70	5·6	+	++

Symbols as Table 2.

more soluble than active proteinase. Inactive α-components in Fraction II were demonstrated by activation with cysteine or methionine. It is not clear whether the enzymes were inactivated during purification or whether inactive precursor was present in the material.

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The Typing of *Salmonella dublin* by Means of Bacteriophage

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SUMMARY: By the use of six phages, 294 (94 %) of 306 strains of *Salmonella dublin* were classified into eleven types. The remaining twelve strains were insusceptible to all phages. Unfortunately, for the purposes of distinguishing strains of epidemiological interest, 66·9 % of the strains belonged to the same type. Acquired phage resistance was responsible for many of the strains being regarded as different phage types.

The phages were also active on *S. rostock*, *S. gallinarum* and on a small proportion of *S. pullorum* strains tested. They were not active on *S. enteritidis*. Three phages possessed a different range of lytic activity despite the fact that Bail's cross-resistance tests suggested that they were identical.

The ability to type a species of *Salmonella* satisfactorily by means of anti-O phages appears to depend mainly on a large proportion of the strains having acquired resistance to different phages.

In view of the importance of *Salmonella dublin* infection in cattle in this country, brought to light mainly by the work of Field (1948, 1949*a*, *b*), and of the fact that *S. dublin* is occasionally responsible for outbreaks of food poisoning in this country, some of which have been traced to infected cows' milk, it was considered worthwhile to search for a method of typing *S. dublin* by means of bacteriophage.

The main investigations were conducted in the hope of finding phages similar to those used by Craigie & Yen (1938) for typing *S. typhi*. Although no such phages were found, it was possible to classify strains of *S. dublin* to some extent with anti-O phages. When this work was nearing completion, Lilleengen (1950) published a classification of *S. dublin* closely resembling mine. The main purpose of this paper is to indicate some of the reasons why it is possible to divide *S. dublin* into types using anti-O phages.

MATERIAL AND METHODS

The methods were exactly those used in investigations on *S. thompson* (Williams Smith, 1951*a*, *b*). All the phages, except one (phage E), were isolated from lysogenic strains of *S. dublin*; phage E was isolated from a culture of *S. dublin* that was actively infected with it. The strains of *S. dublin* were identified by numbers and the phages by letters.

Since pure phage preparations can be obtained only by using non-lysogenic strains for propagation (Williams Smith, 1951*b*) great care was taken to ensure that all propagating strains were non-lysogenic. All the phages were propagated on the same strain, a Type 1 strain.

RESULTS

Twenty phages were isolated from lysogenic strains of *S. dublin.* These phages, together with the phage E, were tested on 50 strains of *S. dublin.* Six of the phages, A–F, were of value in differentiating the strains; the lytic activity of the remainder resembled that of one or other of these six phages. Phages A–D, were active only on smooth strains; phages E and F were active on smooth, and on some rough strains.

The six phages were applied in test dilution to 306 strains of *S. dublin.* Of those 306 strains, 294 (96 %) were typable and 12 (4 %) were insusceptible to the phages. The 306 strains were divisible into eleven types according to the pattern of their reactions with the six phages (Table 1). The Type 1 strains were fully susceptible to all the test phages; the other types were susceptible only to some of these phages. All Types 1–8 were smooth strains. Types 9–11 and the untypable strains were both smooth and rough. Unfortunately, from the point of view of analysing epidemics, 205 (66·9 %) of the strains belonged to Type 1. An extensive search was made for phages that would subdivide Type 1; but none was found.

Table 1. *The phage-typing of* 306 *strains of* Salmonella dublin

	Typing phages						Strains of S. dublin	
Type	A	B	C	D	E	F	No.	%
1	CL	CL	CL	CL	CL	CL	205	66·9
2	CL	CL	CL	CL	CL	—	12	4·0
3	CL	—	CL	CL	CL	CL	17	5·6
4	CL	CL	CL	CL	—	CL	29	9·5
5	CL	—	CL	CL	—	CL	2	0·7
6	—	CL	CL	CL	CL	CL	8	2·6
7	—	CL	—	—	CL	CL	3	1·0
8	—	—	—	CL	CL	CL	8	2·6
9	—	—	—	—	CL	CL	3	1·0
10	—	—	—	—	CL	—	6	3·0
11	—	—	—	—	—	CL	1	0·3
Untypable	—	—	—	—	—	—	12	4·0

CL, confluent lysis; —, no lysis. The phage types have been numbered only for the purpose of this table and are not recognized designations.

Most of the 306 strains were isolated from cows that were either suffering from clinical *S. dublin* infection or were symptomless faecal excreters of this organism. Eight strains were responsible for different outbreaks of food poisoning in human beings; phage-typing did not distinguish them from strains obtained from cows.

Some of the strains were tested several times during a period of 2 years and, apart from the variations inherent in any method of phage-typing depending on pattern reactions, they showed no significant variation in their susceptibility to the test phages. During this period they were subcultured several times.

The significance of lysogenic strains in the phage-typing method

Acquired phage resistance was responsible for many strains being regarded as distinct phage types in the case of staphylococci (Williams Smith, 1948) and *S. thompson* (Williams Smith, 1951*b*). The strains had acquired resistance to different phages in the field—indicated by the fact that they were lysogenic—and were consequently resistant to some of the phages used for typing. A similar state of affairs is possible in the typing of *S. dublin*.

If acquired phage resistance is responsible for the heterogenicity of *S. dublin* strains, lysogenicity would not be a common feature of Type 1 strains, since these strains were fully susceptible to all the typing phages, but it would be in strains belonging to Types 2–11 because these strains were resistant to one or more of the typing phages. Accordingly, 70 Type 1 strains were tested, and none of them were lysogenic; 24 (63 %) of 38 strains belonging to Types 2–11 were lysogenic.

Investigations were carried out in a manner similar to that which has been successful in elucidating the relationship among the staphylococcal and *S. thompson* strains. The principle of the method was to prepare a variant of a Type 1 strain, i.e. a strain showing no evidence of acquired phage resistance, resistant to the phage or phages carried by a lysogenic strain in Types 2–11. The resistant variant and the lysogenic strain were then tested by the typing phages, and, if their reactions were similar, it was concluded that acquired phage resistance was responsible for the originally determined phage type of the lysogenic strain. A young broth culture of the lysogenic strain was heated to 56° for 30 min. and tested for bacteriological sterility. Resistant variants of a Type 1 strain were then made to the phage contained in the heat-treated culture. The phage was not purified because some lysogenic cultures may contain more than one phage. The results of some of these experiments (Table 2) indicated that acquired phage resistance appeared to be responsible for the phage reactions of Types 1–3, 5, 7, 9 and 10. Acquired resistance could also account for the insusceptibility of the untypable strain (i) to all the typing phages and for that of the untypable strain (ii) to some of the phages.

The phages from different strains belonging to one lysogenic phage type all induced the same kind of resistance in Type 1. For example, the five variants of a Type 1 strain resistant to the phages carried by five strains of Type 3 all had the same reactions as Type 3 strains. Again, variants of a Type 4 strain, as well as of a Type 1 strain, were sometimes prepared resistant to the phages carried by lysogenic strains. The resistant variants of a Type 4 strain had the same phage reactions as similar variants of a Type 1 strain except, of course, that they were always insusceptible to phage E, the phage that lysed Type 1 but not Type 4 strains.

No evidence for the origin of Types 4, 6, 8, and 11 was discovered since none were lysogenic. It is known that some artificially-prepared resistant variants may be non-lysogenic, and it may be that these strains had developed phage resistance without becoming lysogenic. Support for this view was found by preparing on four occasions variants of a Type 1 strain resistant to phage E,

Table 2. *The action of the typing phages on lysogenic strains and on variants of a Type* 1 *strain resistant to the phages carried by these lysogenic strains*

The resistant variants of the Type 1 strain are designated by the word Type 1 followed, in brackets, by the type number of the lysogenic strain from which the 'crude' phage was obtained. All the phages were used in test dilution.

	Typing phages					
Strain	A	B	C	D	E	F
Type 1	CL	CL	CL	CL	CL	CL
Type 2	CL	CL	CL	CL	CL	—
Type 1 (Type 2)	CL	CL	CL	CL	CL	—
Type 3	CL	—	CL	CL	CL	CL
Type 1 (Type 3)	CL	—	CL	CL	CL	CL
Type 5	CL	—	CL	CL	—	CL
Type 1 (Type 5)	CL	—	CL	CL	—	CL
Type 7	—	CL	—	—	CL	CL
Type 1 (Type 7)	—	CL	—	—	CL	CL
Type 9	—	—	—	—	CL	CL
Type 1 (Type 9)	—	—	—	—	CL	CL
Type 10	—	—	—	—	CL	—
Type 1 (Type 10)	—	—	—	—	CL	—
Untypable (i)	—	—	—	—	—	—
Type 1 (untypable (ii))	—	—	—	—	—	—
Untypable (ii)	—	—	—	—	—	—
Type 1 (untypable (ii))	—	—	—	—	CL	CL

Symbols as in Table 1.

the phage which distinguishes Type 4 from Type 1. The variants were subcultured twice and then tested for lysogenicity; all were non-lysogenic. However, it is quite possible that factors other than acquired phage resistance accounted for the identity of these four types. One obvious and well-known factor which can account for change in phage susceptibility is smooth → rough variation. In the case of the *S. dublin* strains rough variation was associated with insusceptibility to phages A–D. But not all the strains insusceptible to these phages were rough, however. It should also be remembered that bacteriophage action itself can induce rough variation.

The differentiation of the typing phages by Bail's cross-resistance tests

Cross-resistance tests were carried out according to the method of Bail (1923) in order to determine the relationship of the six typing phages to each other. The resistant variants were prepared from a Type 1 strain. These tests showed that phages B, E, and F differed from each other and from the other phages. Each of the variants resistant to phages A, C and D was completely resistant to all three phages; they were fully susceptible to phages B, E, and F. According to Bail's interpretation, phages A, C and D should therefore be considered identical. The phage-typing results (Table 1), however, do not support this since phages C and D were active, and phage A inactive, on Type 6 strains; whereas phage D was active, and phages A and C inactive, on Type 8 strains.

The action of the Salmonella dublin *phages on other species of Salmonella*

The six phage preparations in test dilution were examined for activity on species of *Salmonella* other than *S. dublin*. The species and number of strains of each species examined were:

Group A: *S. paratyphi* A (2).

Group B: *S. paratyphi* B (2), *typhi-murium* (4), *stanley* (1), *derby* (3), *california* (1), *abortus-equi* (1), *abortus-ovis* (2), *abortus-bovis* (2), and *brancaster* (1).

Group C: *S. paratyphi* C (2), *cholerae-suis* (7), *cardiff* (1), *daytona* (1), *montevideo* (4), *oranienburg* (2), *potsdam* (1), *concord* (9), *barielly* (2), *tennessee* (2), *muenchen* (1), *manhattan* (1), and *düsseldorf* (1).

Group D: *S. typhi* (2), *enteritidis* (20) *rostock* (2), *moscow* (1), *blegdam* (1). *pensacola* (1), *dar-es-salaam* (2), *gallinarum* (12) and *pullorum* (15).

Group E: *S. london*, (1), *give* (1), *anatum* (2), *meleagridis* (1), and *zanzibar* (1).

Other groups: *S. habana* (1), *worthington* (1), *cubana* (1), *kirkee* (1), *kentucky* (1), *carro* (1), *minnesota* (1) and *inverness* (1).

The phages lysed only three of the species tested: *S. gallinarum*, *pullorum* and *rostock*. The twelve strains of *S. gallinarum*, the two *S. rostock* strains and one of the 15 *S. pullorum* strains were fully susceptible to all six typing phages. Two *S. pullorum* strains were lysed by phage F; the remaining 12 *pullorum* strains were resistant. Despite the close antigenic similarity between *S. enteritidis* and *S. dublin*, none of the 20 strains of *S. enteritidis* was susceptible.

DISCUSSION

Acquired phage resistance can account for many strains of *S. dublin* being considered as different phage types. The position is similar to that in staphylococci (Williams Smith, 1948) and *S. thompson* (Williams Smith, 1951*b*).

The different phage types were relatively stable provided, of course, no changes such as rough variation occurred. Whether change of type occurs frequently in the field is another matter. This possibility was discussed previously with regard to *S. thompson* (Williams Smith, 1951*b*) and depends on the opportunity a bacterial population has of becoming phage-infected, acquiring a resistance to the phage with which it has become infected and, as a result, undergoing a change in type. Though such a change might occur during epidemiological investigations conducted over a long period of time, the possibility of its occurrence during a short-term inquiry would be fairly remote.

An important limitation to the use of phage for typing *S. dublin* is that 66·9 % of the strains examined belonged to one type; membership of this type would be of little significance from the epidemiological point of view. This fact is related to the observation that most of the strains were non-lysogenic, exhibited no evidence of acquired phage resistance, and, consequently, were fully susceptible to all the typing phages. *S. thompson* differed (Williams Smith, 1951*b*), since 84 % of the strains examined were lysogenic and could be classified into eleven types, the largest comprising only 28 % of the whole. In the case of *S. cholerae-suis* (Williams Smith, 1951*b*) 88 % of the strains were lysogenic, but this did not permit a satisfactory classification because these strains were latently infected with the same phage and, consequently, exhibited the same degree of phage resistance. It is clear, therefore, that to classify a species of Salmonella satisfactorily by anti-O phages, not only should a high proportion of strains be lysogenic but many of the phages they carry should be different.

Lilleengen (1950) also found that the majority (62 %) of the 129 strains of *S. dublin* he examined were fully susceptible to all his typing phages. The remaining types were distinguished from each other, as in the present work, by their pattern of phage reactions. Lilleengen's phages were obtained by the filtration of such material as sewage and faeces, whereas the phages in this work were isolated from lysogenic strains of *S. dublin*. The phages from the lysogenic strains appear to be more specific in their action than those from sewage or faeces, since none of them were active on *S. enteritidis* and only one of 15 strains of *S. pullorum* was fully susceptible to them; the phages used by Lilleengen were active on most strains of *S. pullorum* tested and some of them were active on *S. enteritidis*.

The cross-resistance tests substantiate previous doubts about the accuracy of such tests (Burnet, 1929; Williams Smith, 1951*b*). The three phages, A, C and D, were considered identical when a Type 1 strain was used as a 'parent' strain for carrying out cross-resistance tests but were shown to have a different range of lytic activity when tested on the series of strains of *S. dublin*. It is conceivable that the variants of the Type 1 strain-resistant to any one of these three phages also lacked receptors for the other two, but that some strains such as those of Types 6 and 8 possessed receptors which were susceptible to attack by some but not all of the three phages. It is noteworthy that no evidence was obtained to suggest that acquired phage resistance was responsible for the identity of Types 6 and 8. No other reasons for their identity were found.

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Isolation of Sexual Strains of *Chlamydomonas*

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SUMMARY: The zygospores of several species of *Chlamydomonas* present in dry soil are very resistant to dehydrating agents. Based on this resistance a method for the isolation of sexual strains has been developed, and two homothallic and three heterothallic species of *Chlamydomonas* have been obtained in pure culture.

A unicellular green alga which can grow without difficulty in pure culture, and which can be induced to undergo a complete sexual life cycle under experimental conditions, would offer possibilities as a suitable organism for an attack on problems in biochemical genetics for which *Neurospora* and similar heterotrophs are unsuited. Mating strains of a few species of *Chlamydomonas* have been isolated (Moewus, 1933; Gerloff, 1940; Smith, 1946; Bold, 1949; Lewin, 1949), but for various reasons none has proved altogether satisfactory.

Several workers experienced difficulty in obtaining sexual strains when random isolates of *Chlamydomonas* from soil were paired and examined for mating reactions. Schreiber (1925) reported a lack of success when as many as 70 clones were tested in this way. An easier method was therefore sought and the following procedure, based chiefly on the differential sensitivity of spores and vegetative cells to desiccation, was devised.

Zygospores of *C. moewusii* Gerloff are only produced as a result of sexual fusion of gametes; this is probably true also for most species in this genus. Such spores, which are only slightly more resistant to heat than are vegetative cells, are nevertheless remarkably resistant to desiccation. The walls are so impermeable that spores may remain viable after immersion for a week in acetone, whereas this treatment kills vegetative cells within a few seconds. Bold (1949) isolated mating types of *C. chlamydogama* Bold from air-dry soil; Smith (1948) stated that from his experience 'dry soil is a better source of sexual material than is damp soil'. It seems probable that the mating strains obtained by these authors arose from such drought-resistant zygospores.

METHOD

An enriched soil culture containing vegetative cells and zygospores is treated with acetone. From surviving spores a crude culture is grown. This consists of algal strains presumably capable of again producing zygospores. Pure cultures of these can be obtained by the usual method of dilution streaking on agar. When tested, they may prove either to be homothallic, producing zygospores within the clone, or they may exhibit heterothallism, and only form zygospores when mixed with cells from a culture of opposite mating type. Finally, bacteria-free cultures can be obtained. Details of a preliminary experiment using this method are given below.

Experimental procedure

Special difficulties in temperature control arise when attempting laboratory culture under artificial illumination of algae sensitive to heat. As sources of inocula, therefore, soils were chosen from comparatively warm environments, ten from Arizona (from a collection made in 1950 by Dr John Reeder, Osborn Botanical Laboratory) and one from a heated greenhouse in New Haven, Connecticut. These samples were treated in the following manner.

Preparation of enrichment cultures. About 2 g. soil were put into 30 ml. of a mineral medium satisfactory for the rapid growth of *Chlamydomonas* and other algae. This medium had the following composition: $Ca(NO_3)_2.4H_2O$, 1·0 g.; $MgSO_4.7H_2O$, 0·2 g.; K_2HPO_4, 0·2 g.; trace elements (see Burkholder & Nickell, 1949). The use of any organic supplement such as soil extract was avoided, in order to discourage the growth of micro-heterotrophic organisms, such as *C. chlamydogama* (Hutner & Provasoli (in press)), the organic growth-substance requirements of which would have to be determined before critical physiological studies could be undertaken.

The flasks so prepared were illuminated under batteries of 'daylight' fluorescent lamps at *c.* 500 f.c. intensity, at 23°, for 2 weeks. During this period a dense growth of mixed algae developed, and zygotes were formed by such species as were capable of growth and of the production and fusion of gametes under these conditions.

Acetone treatment. At the end of a fortnight, the contents of each flask were mixed by shaking. Five ml. of the algal suspension was mixed with an equal volume of acetone, allowed to stand for 10 min., and then filtered. The residue was drained and dried in air for 20 min., to permit evaporation of the acetone, and then rinsed into flasks containing 30 ml. of sterile mineral medium, which were then incubated and illuminated as before.

Isolation of strains derived from acetone-resistant zygospores. After illumination for 11 days, seven of these flasks showed abundant growth of unicellular green algae, presumably derived from acetone-resistant zygospores. This period of growth was not extended, since it is preferable for most experimental purposes to work with species in which there is not a prolonged period of zygospore dormancy, as there is normally in *C. moewusii* (Lewin, 1949). With a flamed bacteriological loop, streaks were made from each of the seven flasks on Petri plates containing mineral medium solidified with 1 % agar. These plates, kept inverted to eliminate condensation of water on the lid, were incubated under continuous illumination.

Within a week, colonies were visible to the naked eye. Five well-isolated colonies were selected on each plate, and transferred to tubes containing *c.* 5 ml. of sterile mineral medium. (One of the plates had two types of colonies: the smaller proved to consist of *Chlorococcum* sp., and will not be considered further here.) After 5 days of illumination, each tube contained a fairly heavy suspension of algal cells of the *Chlamydomonas* type. The period of growth was kept short and the medium used simple, since species which manifest a sexual response only after a long period of vegetative development, or under specifically altered nutrient conditions, were not desired.

Mating tests. Within each set from the seven flasks, the five supposedly clonal cultures from the selected colonies were then tested for mating in all combinations in checker-board fashion, 5 × 5, by mixing drops directly on the surface of a mineral agar plate. The drops were allowed to fall from pipettes. While there was no attempt at aseptic technique at this stage, the pipettes stood in boiling water between transfers, and care was taken to eliminate involuntary cross-contamination of algae. The use of plates prepared 2 days previously, in which the agar surface had dried somewhat, decreased the danger of fusion between neighbouring droplets. The plates were illuminated for 5 days.

In the author's experience, immediate microscopic examination of such mixtures for the presence of clumping or pairing cells was very time-consuming. As mating had in all cases to be confirmed by the formation of zygospores, no preliminary microscopic examination was made, and ultimate spore-formation was used as the criterion for sexual activity. One Petri dish served for each set, the positions of the various combinations being oriented with the help of a guide mark on the base of the plate.

After 5 days on each plate twenty-five green patches were then apparent, comprising colonies of the original five clones and, in duplicate series, the ten possible paired combinations. These colonies were scanned under a magnification of × 100, and scored for the presence or absence of zygospores. Samples were also mounted on slides and checked under a higher magnification.

Preparation of bacteria-free cultures. The cultures which were considered of interest, after testing for sexual reactions as above, were obtained in bacteria-free condition by dilution streaking on agar plates. (One culture, which by itself produced zygospores when first isolated, proved on subsequent examination to have been a mixture of complementary mating types.) Cultures were checked for bacterial contamination in a liquid medium containing glucose (1 %) and Bacto-tryptone (Difco) (0·5 %), and have since been maintained on agar slants containing minerals, sodium acetate (0·1 %) and Bacto-tryptone (0·5 %). From such stocks, liquid cultures in mineral medium were prepared, and rechecked for mating behaviour. In addition, cultures from different soil samples were tested for mating with one another, in order to establish possible specific identities.

RESULTS

The alga obtained from New Haven greenhouse soil proved to be homothallic, as did one from Arizona. Of the five others which survived the acetone treatment, one yielded no zygospores in any mating combination (even if both mating types had been present, mating conditions may have been otherwise unfavourable). The other four showed typical heterothallic responses. Two isolates from neighbouring sites proved cross-fertile when suitably combined.

Thus mating types of three heterothallic species of *Chlamydomonas*, and cultures of two homothallic species, were obtained from eleven soil samples, demonstrating the practicability of the method described.

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PROOM, H. & WOIWOD, A. J. (1951). *J. gen. Microbiol.* 5, 930–938.

Amine Production in the Genus *Proteus*

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SUMMARY: All of 43 strains of *Proteus vulgaris*, 75 strains of *P. mirabilis* and 10 strains of *P. morganii* when grown in an acid-hydrolysed casein or nutrient broth medium produced *iso*butylamine and two other amines tentatively identified as *iso*amylamine and β-methylbutylamine. These amines were not produced by any of 239 strains representative of other species in the family Enterobacteriaceae grown under the same conditions. This character is sufficiently constant to be included in the generic description. Ten strains of *P. rettgeri* did not produce these amines, and for this and other reasons it is proposed that this species be excluded from the genus *Proteus*.

The extension of the genus *Proteus* by Rauss (1936) to include the relatively slow urea-decomposing species *P. morganii* and by Rustigian & Stuart (1943) to include the anaerogenic mannitol-fermenting species *P. rettgeri* increases the difficulty of distinguishing between *Proteus* spp. and non-lactose-fermenting strains which are probably more closely related to other genera. A test specific for *Proteus* spp. therefore would be of value in defining this genus. We have shown (Proom & Woiwod, 1949) that when *Proteus* spp. are grown in an acid-hydrolysed casein medium a characteristic change occurs on the developed chromatograms of the cultures. This change consists in the appearance of two ninhydrin-positive spots which have R_F values greater than leucine on the single dimensional chromatogram using *n*-butanol+acetic acid as the solvent. We suggested that these ninhydrin-positive substances were polypeptides. We have confirmed that on acid 'hydrolysis' these substances produce amino-acids, but the deduction that therefore they were polypeptides was incorrect. As will be demonstrated, these substances are amines and amine production is characteristic of the genus *Proteus*.

EXPERIMENTAL

Amine production by Proteus

When counter-current extraction methods were used in an attempt to isolate from *Proteus* cultures the two new substances which appeared on the developed chromatogram these substances were often lost when solutions containing the separated fractions were evaporated to dryness. This suggested the substances were volatile, and it was found that they could be separated from the culture filtrates by steam distillation at alkaline reaction.

A strain of *Proteus* was grown on an acid-hydrolysed casein medium (Proom & Woiwod, 1949). Seven litres of culture filtrate were adjusted to pH 9·0 and steam-distilled into 0·1 N-HCl, and the distillate dried on a water-bath in a vacuum. The material was made alkaline and extracted with ether, and the ether was removed by heating on a water-bath. The weight of the dried residue was 1·5 g. This was dissolved in 10 ml. distilled water saturated with *n*-butanol and distributed between *n*-butanol and distilled water in an all-glass 27-unit Craig separator (Craig & Post, 1949). The distribution was studied by paper chromatography. The two components were almost completely separated after one hundred transfers. The respective solutions containing the two separated components were separately bulked and evaporated to dryness in a vacuum. The yields were approximately 130 mg. of the slower running material and 350 mg. of the faster running material.

The slower running substance proved to be *iso*butylamine. It was compared with an authentic sample of *iso*butylamine and behaved identically when run on chromatograms using several solvents. The oxalate derivative was prepared and it had the same melting-point (167° uncorr.) as the oxalate prepared from the authentic *iso*butylamine. The mixed melting-point of the two derivatives was not depressed. This amine is probably derived from valine.

A number of *Proteus* strains were grown on a basal medium containing inorganic salts, ammonium chloride and glucose with nicotinic and pantothenic acids added as essential nutrients. Under these conditions the amines were not produced. The simplest medium in which *Proteus* spp. regularly produced only the slower running substance was the basal medium to which glutamic acid and valine had been added. In a few instances a little of the slower running substance was produced by the addition of valine only. No amines were produced by the addition of glutamic acid only.

The faster running substance was not completely identified but seems likely to be a mixture of *iso*amylamine and *β*-methylbutylamine produced from leucine and isoleucine respectively. The isolated fraction was compared chromatographically with an authentic sample of *iso*amylamine and could not be distinguished from it with any of the several solvents used. Strains of *Proteus* spp. grown on the previously described basal medium with added glutamic acid and leucine or isoleucine produced only the faster running substance. The intensity of the faster running spots on the developed chromatograms from cultures grown on the medium with added isoleucine was much less than those from the medium with added leucine, suggesting that the major constituent of the faster running substance was derived from leucine. A sample of *β*-methylbutylamine was not available, but it seems unlikely that it could be easily distinguished from *iso*amylamine by chromatographic methods. Attempts to characterize the faster running material by the preparation of derivatives failed and we attribute this failure to the probability that the material was a mixture of these two amines. The amine nature of this material is evident from the fact that it is volatile at alkaline reaction and gives a positive ninhydrin reaction. The chromatographic evidence strongly supports the view that it is a mixture of *iso*amylamine and *β*-methylbutylamine produced from leucine and isoleucine respectively.

Both the slower and faster running materials were eluted from paper chromatograms and acid hydrolysed. The hydrolysate was examined chromatographically, and a number of ninhydrin-positive spots were obtained. The observation reported previously (Woiwod & Proom, 1950) was confirmed, but there can be little doubt that these two substances are amines and not polypeptides as was assumed from the hydrolysis experiments. The mechanisms responsible for these reactions are not known. This is the second time we have observed unexpected chemical reactions occurring under these conditions, the first instance being the production of a number of substances from γ-amino-butyric acid (Woiwod & Proom, 1950).

It was found that after drying a mixture of glucose and *iso*butylamine or glucose and *iso*amylamine and chromatographing the dissolved residue, strong ninhydrin-positive reactions were obtained in positions corresponding to valine and leucine respectively. It is possible that carbohydrate material eluted from the paper is in part responsible for the observed effects. It is clear that the appearance of fresh ninhydrin-positive substances after treatment with hydrochloric acid cannot be taken as unequivocal evidence of the presence of peptides. All our experiments were controlled by the hydrolysis of eluates from blank paper, and the observed effects could not be attributed to peptides originally present in the paper.

Chromatographic analysis of Proteus *cultures*

Strains were grown for 3 days at 37° in an acid-hydrolysed casein medium (Proom & Woiwod, 1949), the medium being dispensed in 2 ml. amounts in ¼ oz. screw-capped bottles. After incubation the bottles were steamed for 30 min. and the cultures examined by the chromatographic technique described by Woiwod (1949).

The chromatograms of the cultures of all the 128 strains of *Proteus* examined showed the characteristic ninhydrin-positive spots. Amine production was therefore demonstrated in all the *Proteus* cultures. Of these strains 43 were *P. vulgaris*, 75 *P. mirabilis* and 10 *P. morganii*. These strains had been isolated from a variety of sources and covered a wide range of serological types.

We also examined 50 strains of each of the genera *Salmonella*, *Shigella* and *Escherichia*, 25 strains of *Aerobacter*, 9 strains of *Klebsiella*, 7 strains of *Serratia*, 6 strains of *Erwinia*, 40 strains of uncertain taxonomic status, including many paracolon types, and 10 strains of *P. rettgeri*, some of which were received from the National Collection of Type Cultures (N.C.T.C. 7475–81), two from this collection under the name of *Bacterium kandiense* (N.C.T.C. 3162, 3163) and one fresh isolate. In no instance was the production of amines observed by the chromatographic technique.

Ninhydrin paper test for the presence of amines

Since primary amines are volatile at alkaline reaction and react with ninhydrin at room temperature to give a purple colour, it was possible to develop a simple test for the presence of amines which is suitable for routine bacteriological work. After considerable preliminary investigation the following conditions were found to be suitable for the test.

The cultures are grown for 24 hr. at 37° in 4 oz. flat screw-capped bottles (United Glass Bottle Co.) containing 20 ml. nutrient broth. A sheet of Whatman no. 54 filter-paper is soaked in a 0·5 % (w/v) solution of ninhydrin in *n*-butanol and dried at room temperature for 15 min. An approximate 1·5 cm. square is cut from the paper and placed between an extra rubber liner and the bottle cap, both liner and cap being perforated centrally with a hole about 0·5 cm. diameter, The cap is removed from the culture bottle and three drops of capryl alcohol, 2 ml. 10N-NaOH and about 0·5 g. aluminium powder (British Drug Houses Ltd., coarse, grease-free) is added. The cap containing the ninhydrin paper is then screwed on the culture bottle which is left at room temperature. The bottle is gently shaken to produce a vigorous flow of hydrogen. The hydrogen carries the amines through the filter-paper where they react with ninhydrin to give a purple colour. This colour develops at room temperature, usually within 15 min., and reaches maximum intensity within 1 hr. With broth controls and cultures which do not produce amines the filter-paper remains white. Some cultures produce a considerable amount of ammonia and this may react with ninhydrin to give a yellow colour, but this change is seldom observed within the first 15 min. The intensity of the purple colour varies with the strain, but all the 128 strains of *Proteus* tested under these conditions gave an unequivocal purple within 15 min. All of 50 strains representative of other species in the family Enterobacteriaceae gave a negative reaction. Although it is unnecessary to remove the cap in order to read the test, the purple colour appears very much more intense when viewed by transmitted light.

We found it necessary to adhere fairly closely to this technique. When the concentration of ninhydrin used for the test paper is decreased much below 0·5 % the test becomes insensitive, and when the concentration is raised above 1·0 % the controls may show a reddish tinge. The *n*-butanol solution of ninhydrin keeps well, but papers should be freshly prepared for each test. When this test is performed with smaller quantities of culture, i.e. 5 ml. in 1 oz. screw-capped bottles, the amount of amine produced by some strains is not sufficient to give a good positive result. Sufficient space must be left between the surface of the culture and the cap since, in spite of the capryl alcohol, considerable frothing occurs, and if froth reaches the ninhydrin paper, false positive reactions occur. In spite of the considerable evolution of hydrogen no trouble was experienced with spray reaching the filter-paper and thus giving false positive reactions. No advantage is gained by developing the colour by heating the cap in a hot air oven; some test papers develop a purplish tinge on prolonged heating. When a number of cultures are being examined the caps may be removed and the capryl alcohol and alkali added to a row of bottles, but after adding the aluminium powder the test caps must be screwed on within a few secounds, otherwise considerable loss of amine occurs.

Nutritional requirements of Proteus rettgeri

Since all our ten strains of *P. rettgeri* failed to produce amines it was necessary to reconsider the taxonomy of this species, and it was felt that nutritional studies would be of value. The nutritional requirements of these strains were determined by the methods described by Knight & Proom (1950).

All 10 strains grew in a medium in which ammonia and a number of synthetic amino-acids were the only source of nitrogen and without the addition of growth factors.

It is well established that a requirement for nicotinic acid is a characteristic property of *Proteus* spp. Fildes (1938) first demonstrated this requirement for *P. vulgaris*, and Pelczar & Porter (1940*a*) for *P. morganii*. The latter organism also requires pantothenic acid as an essential nutrient. Quite large numbers of strains have been examined; for example, Pelczar & Porter (1940*b*) examined 169 strains of *P. vulgaris* and *P. mirabilis*, and only 5 of these strains did not require nicotinic acid.

To check our methods and as additional confirmation of the well-established observations we examined 76 strains of *Proteus*, including representatives of the species *vulgaris*, *mirabilis* and *morganii*. It was considered justifiable for this purpose not to distinguish between the requirements for nicotinic acid and for pantothenic acid. The strains were therefore grown on two media, one containing hydrolysed casein and without growth factors and a second similar medium with added nicotinic and pantothenic acids. Of the 76 strains 73 failed to grow on the hydrolysed casein medium but grew on this medium with added nicotinic and pantothenic acids. Of the three anomalous strains one required the growth factors when the hydrolysed casein was replaced by a mixture of seven amino-acids (asparagine, alanine, glutamic acid, leucine, methionine, proline and serine) and the remaining two required the growth factors when the hydrolysed casein was replaced by ammonia.

Biochemical reactions

The more important diagnostic biochemical reactions of our *Proteus* strains were determined. The fermentation reactions were determined in ¼ oz. screw-capped bottles containing *c.* 3 ml. of 1·0 % peptone water + 0·5 % of the relevant carbohydrate, with bromocresolpurple as indicator. H_2S production was determined by growth in 10 ml. nutrient broth in cotton-wool plugged test-tubes with a strip of lead acetate paper as the indicator. Indole production was determined by Kovács's (1928) method, using amyl alcohol as the solvent. Urea decomposition was determined by Christensen's (1946) method. Amine production was determined by both the chromatographic and the ninhydrin test-paper methods.

The results of the biochemical tests are summarized in Table 1. These tests were performed primarily to check the identity and purity of our cultures. The strains have been classified according to *Bergey's Manual* (1948), since this appears to be the simplest classification. Those strains which failed to ferment sucrose or to produce indole were classified as *P. mirabilis*, although a number of such strains were received as *P. morganii*. H_2S production was marked and occurred within 24 hr. with the species *vulgaris*, *mirabilis* and *morganii*, but was weak and slow with *rettgeri*, taking 4 days. There were a few deviations from the typical patterns for each species. A number of strains were tested for gelatin liquefaction, primarily to confirm that this character is variable within the group. Of the 46 strains of *P. vulgaris or P. mirabilis* examined, one strain of *P. vulgaris* and five of *P. mirabilis* failed to liquefy gelatin, the rate of liquefaction being variable and showing little correlation with species. All 10 strains of *P. morganii* failed to liquefy gelatin.

Table 1. *Biochemical reactions of* Proteus *species*

		Fermentation of					Production of			
Species	No. of strains tested	Glucose	Lactose	Mannitol	Maltose	Sucrose	Indole	H_2S	Amines	Urea decomp.
P. vulgaris	43	AG	–	–	AG	AG	+	+	+	+(1)
P. mirabilis	75	AG	–	–	–	AG late or –	–	+	+	+(2)
P. morganii	10	AG	–	–(1)	–	–	+	+	+	+
P. rettgeri	10	A*	–	A	–	A slow or –	+	±	–	+

AG = acid and gas; A = acid; + = positive; – = negative.
The bracketed figures give the numbers of strains which gave anomalous reactions.
* One strain of *P. rettgeri* produced a little gas from glucose.

DISCUSSION

The genus *Proteus* represents a fairly well-defined group within the peritrichously flagellated Gram-negative rods. Hauser (1885) first described the genus, noting the characteristic swarming on agar plates. He named two species, *P. vulgaris* and *P. mirabilis,* which were distinguished by speed of gelatin liquefaction. Smith (1894) described the fermentation by *Proteus* spp. of glucose and sucrose with production of small volumes of gas, and the failure to ferment lactose. These reactions were regarded as characteristic of the genus. Wenner & Rettger (1919) divided their cultures into two species: *P. vulgaris* which fermented maltose and *P. mirabilis* which did not. Moltke (1927) demonstrated that his cultures of *Proteus* rapidly hydrolysed urea. Rauss (1936) examined a number of cultures of Morgan's bacillus no. 1, and on biochemical and cultural grounds included this species in the genus *Proteus.* This inclusion is now generally accepted. Rustigian & Stuart (1943), from a study of 48 strains of *Bacterium rettgeri* (Hadley, Elkins & Caldwell, 1918) concluded that this species should be included in the genus *Proteus.*

Bergey's Manual (1948) divides the genus into four species, *P. vulgaris, P. mirabilis, P. morganii* and *P. rettgeri,* on the basis of fermentation of maltose, sucrose and mannitol and the production of indole. *Topley & Wilson's Principles* (1946) gives the failure to ferment mannitol and ability to produce H_2S as generic characters, and divides the genus into a number of subgroups on the basis of fermentation of maltose and sucrose, indole production, rate of hydrolysis of gelatin and serum, and peptonization of milk. Specific names are not ascribed to these subgroups nor is there reference to the species *rettgeri.*

Fulton (1943) examined 18 strains of *Bacterium columbense* (Castellani), and suggested that these organisms should be classified together with Morgan's bacillus no. 1 into a new genus *Morganella.* Our examination of six strains of

Bact. columbense received from the National Collection of Type Cultures (708, 4647, 2702, 5254, 5497, 5498) is in agreement with the description given by Fulton. We consider this species a paracolon organism; apart from its failure to ferment lactose it closely resembles *Escherichia coli.* None of these six strains of *Bact. columbense* produced amines. We think that no useful purpose would be served by creating a separate genus for Morgan's bacillus and that this would obscure the close relationship which exists between this species and other members of the genus *Proteus.*

Rustigian & Stuart (1943, 1945) stated that *P. morganii* and *P. rettgeri* do not produce H_2S and the inability to produce H_2S is included in the description of these two species given in *Bergey's Manual* (1948). Rustigian & Stuart (1945) appreciated that many authors have reported H_2S production by *P. morganii* and suggested that this discrepancy was due to the methods used and the interpretation of the results. They did not describe the method they used. We have found that with the lead acetate test-paper technique *P. morganii* is an active producer of H_2S and *P. rettgeri* a feeble producer of H_2S and we are of opinion that H_2S production is a characteristic property of both the genus *Proteus* and the species *P. morganii.*

We have shown that the ability to produce amines distinguishes *P. vulgaris, P. mirabilis* and *P. morganii* from other species in the family Enterobacteriaceae and we feel that this character is sufficiently constant and characteristic to be included in the genic description. The classification of the species *rettgeri,* does however, require reconsideration. Rustigian & Stuart (1943) placed this organism in the genus *Proteus* for the reason that it has fermentation reactions similar to those of other *Proteus* spp. except that it is anaerogenic and ferments mannitol; it is an active producer of urease and under suitable conditions may swarm in a manner reminiscent of *P. morganii.* The evidence against the classification of *rettgeri* in the genus *Proteus* would now appear to be strong. The species is anaerogenic, ferments mannitol, does not produce amines and does not require nicotinic acid as an essential nutrient.

We consider this evidence sufficient to exclude *rettgeri* from the genus *Proteus.* If *rettgeri* be included it follows that with non-lactose-fermenting strains of Enterobacteriaceae inclusion in the genus *Proteus* depends virtually on the single qualitative character of marked decomposition of urea. Although techniques for urease production, such as those described by Rustigian & Stuart (1941) and Christensen (1946), effectively differentiate between the marked decomposition of urea characteristic of *Proteus* species and of *rettgeri* on the one hand, and the less active decomposition of urea by other species such as *Aerobacter,* nevertheless this difference is still only quantitative. Moreover, an occasional strain of *Proteus* fails to decompose urea. On the other hand, if our subsequent definition of the genus *Proteus* be accepted, then the classification depends on a number of characters, i.e. the fermentation of glucose with small gas volumes, failure to ferment lactose or mannitol, marked decomposition of urea, production of H_2S, production of amines and a nutritional requirement for nicotinic acid. The genus then represents a well-defined and clearly recognizable group of organisms. We do not propose to suggest an alternative classification of *Bact. rettgeri*, since this is part of the problem of the classification of the family Enterobacteriaceae as a whole, and is outside the scope of this paper.

The proposed description of the genus *Proteus* is a modification of that given by Borman, Stuart & Wheeler (1945). The modification consists of including failure to ferment mannitol, production of H_2S, production of amines and a requirement for nicotinic acid as generic characters. The definition is as follows:

Gram-negative, non-sporing rods. Pleomorphic in young cultures. Usually actively motile at 25°; motility may be weak at 37°. Flagella peritrichous when present. Aerobic, poor growth when grown under anaerobic conditions. Motile forms may produce characteristic swarming on nutrient agar. Ferments glucose, but not lactose or mannitol, with the production of small volumes of gas. Decomposition of urea, marked. H_2S *produced. Amines produced. Nicotinic acid is required as an essential nutrient.*

Type species—Proteus vulgaris (*Hauser*)

We are indebted to Prof. B. C. J. G. Knight for help with the nutritional studies, to Mr E. Harris for technical assistance, and to Drs A. A. Miles and F. Kauffmann for most of the strains of *Proteus* examined.

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Pseudomonas aeruginosa—its Characterization and Identification*

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SUMMARY: The species *Pseudomonas aeruginosa* is defined more precisely, and a method is presented whereby apyocyanogenic strains of this species can be correctly identified. This method depends upon the correlated characteristics: (1) ability to grow at 41° ± 1; (2) ability to oxidize potassium gluconate, in shaken culture, to a reducing compound presumed to be potassium 2-ketogluconate; (3) production of slime in static culture in a medium containing potassium gluconate as the principal carbon source.

All strains of *P. aeruginosa* tested, regardless of their pigment-forming capacity or their stage of growth, could be converted to a type of growth characterized by profuse sliminess in liquid potassium gluconate medium.

Detection of pyocyanogenic capacity is shown to be equally reliable in Gessard's glycerol peptone agar and in Burton's defined medium when negative results in one or the other medium are rechecked in both, and when the observation period in Burton's medium is lengthened.

According to the International Bacteriological Code of Nomenclature (Buchanan, St John-Brooks & Breed, 1948), the type species of a genus is the species with which the genus name is permanently associated. The proper characterization of such type species is, therefore, most important. Furthermore, the systematic relationships of related organisms can hardly be made clear until type species are well enough characterized so that they can be readily differentiated. *P. aeruginosa* (Schroeter) Migula, the type species of the genus *Pseudomonas*, is a case in point.

Because of the ease of identification of pyocyanine, the blue-green water- and chloroform-soluble pigment produced by typical strains, *P. aeruginosa* is among the easiest of bacteria to identify. Atypical strains which fail to produce this pigment are common, however, and have been discussed by Lehmann, Neumann & Breed (1931), Gessard (1919), Jordan (1899) and Gaby (1946). In fact, Gaby (1946) found that non-pyocyanine-producing types predominate. He suggested that they are more typical of the species than the more striking pyocyanine-producing types. Furthermore, pyocyanine-producing strains have been reported commonly to lose the capacity to produce this pigment (Jordan, 1899; Hadley, 1927; Lehmann, Neumann & Breed, 1931; Gaby, 1946). The certain identification of such apyocyanogenic strains depends largely upon their history of having once been able to produce pyocyanine, or upon the fact of their isolation from pathogenic processes in higher animals. However, organisms identified as *P. aeruginosa* have been isolated from a variety of other sources including soil, water, sewage, normal skin, and contaminated media. Thus strains not producing pyocyanine, from whatever source, cannot be dismissed from consideration as *P. aeruginosa*.

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As early as 1899, Jordan suggested that the chief difference between *Bacillus fluorescens liquefaciens* (*Pseudomonas fluorescens*) and his fluorescigenic, non-pyocyanine-producing variety of *P. aeruginosa* was one of temperature optima, and perhaps in their behaviour in the animal body. Although he dismissed the hypothesis for lack of convincing evidence, he mentioned the possibility that *P. fluorescens* might be a modified or degenerate form of *P. aeruginosa*. This theory has been advanced time and again over the years, most recently by Sandiford (1937), Christie (1948) and Gaby (1946). Since it could neither be proved nor disproved, it has clouded all attempts to distinguish between the two. Preoccupation with this problem perhaps accounts for the preponderance of attention given in the literature to distinguishing between *P. aeruginosa* and *P. fluorescens*.

Many bacteriologists seem unwilling to admit the existence of other distinct species. The consensus of opinion is probably expressed by Stanier (1947), who suggested the term '*Pseudomonas fluorescens* species group' for fluorescent pseudomonads unable to manufacture accessory phenazine pigments.

Although most serological studies have been unproductive in contributing to a differentiation of species in the genus, Munoz, Scherago & Weaver (1949) have recently found it possible to differentiate serologically among 10 species.

Only two characteristics other than antigenic make-up of the cells have shown much promise. They are growth temperatures and ability to haemolyse blood. The ability of *P. aeruginosa* to grow well at 37°, in contrast to the inability of *P. fluorescens* and most other fluorescent bacteria to do so, is well known and has served as the main differential characteristic for many years. The existence of pseudomonads other than *P. aeruginosa* which grow well at 37°, however, has limited the usefulness of this knowledge. In recent years it has been claimed that the ability to haemolyse blood of certain warm-blooded animals is characteristic of *P. aeruginosa* (Christie, 1948; Reid, Naghski, Farrell & Haley, 1942; Salvin & Lewis, 1946; Gaby, 1946). This knowledge is of limited usefulness, however, in the absence of information to show that other pseudomonads which grow at 37° fail to haemolyse blood.

Differentiation of *P. aeruginosa* from other fluorescent bacteria based upon temperature relationships has been improved by Seleen & Stark (1943) who showed that good growth of *P. aeruginosa* occurs at 42°. The data obtained during the current investigation confirm the view that all strains of this organism grow well at 42°, and in addition demonstrate that this characteristic will, in company with two others, permit the identification of *P. aeruginosa* even though the ability to produce pyocyanine be lacking.

METHODS

Pyocyanine production

Four media were used in surveying the group for ability to produce pyocyanine: Gessard's glycerol peptone agar stabs, (1891; see also Seleen & Stark, 1943), Sullivan's K and M media (1905), and the complete medium of Burton, Campbell & Eagles (1948). Sullivan's K and M media were soon discarded, since only 50 % of strains which produced pyocyanine in Gessard's stabs and in Burton's complete medium produced the pigment in these substrates. Furthermore, no strain which failed to produce pyocyanine in Gessard's medium succeeded in doing so in Sullivan's media.

Burton's medium, dispensed 24 ml. to each 300 ml. flask, was incubated at 30° (air temperature). The larger quantity of medium is a departure from the method as recommended by Burton *et al.*, but was found necessary in order to make tests possible beyond the 4-day incubation period recommended. Many strains required a longer period to produce appreciable quantities of pyocyanine. Tests in Gessard's medium in 16 mm. test-tubes were conducted in a 36° ± 1 water-bath because it has been shown that a more uniform temperature is thus maintained throughout the tube (Gordon & Smith, 1949).

When the intensity of blue or blue-green colour in the medium indicated that a strain had reached its limit of pyocyanine production, the pigment was extracted with chloroform. In the solid glycerol-peptone medium of Gessard the agar was macerated by means of a glass rod to obtain more intimate contact between it and the chloroform. The chloroform containing the extracted pigment was then decanted into another test-tube. In the liquid medium, the supernatant was aspirated and discarded after chloroform extraction. Approximately 1 ml. of distilled water was then added to the chloroform extract in either case to serve as a vehicle for the acid pyocyanine, which was obtained by the addition of 1 drop of N-H_2SO_4. Upon shaking, the pyocyanine became red and insoluble in the chloroform. The appearance of a red water-soluble pigment in the aqueous acid layer was accepted as confirmation of the presence of pyocyanine.

Temperature relationships

All 52 strains were streaked in quadruplicate on agar slants of the following composition (TGY slants): tryptone, 5 g.; glucose, 1 g.; yeast extract, 5 g.; K_2HPO_4, 1 g.; agar, 15 g.; distilled H_2O, 1000 ml.; pH 6·8–7·0.

Slants of each strain were then incubated at each of the following temperatures: 28–30° (air temperature), 36° ± 1 (water-bath temperature), 41° ± 1 (water-bath temperature) and 45° ± 1 (water-bath temperature). Incubation was continued until maximum growth occurred (usually about 48 hr.), or for 1 week.

Oxidation of potassium gluconate

Each strain was inoculated from a stock slant to a 300 ml. Erlenmeyer flask containing 100 ml. of sterile medium of the following composition: tryptone, 1·5 g.; yeast extract, 1 g.; K_2HPO_4, 1 g.; potassium gluconate, 40 g.; distilled water, 1000 ml.; pH 7·0.

The inoculated flasks were then put on a rotary shaker (Gump-type) at 28–30°. The shaker was set at 200 r.p.m. and each flask was rotated through an orbit of 2¼ in. diameter. At 2, 4, 7 and 14 days, samples were tested in the following manner.

To 1 ml. of the culture in a 16 mm. test-tube, 10 ml. of a copper sulphate sugar reagent (Shaffer & Hartmann, 1921—'Carbonate-citrate reagent for cupric titration') were added. The contents of the tubes were thoroughly mixed, heated for 10 min. in boiling water, rapidly cooled in cold water and then set aside until the next morning to settle the precipitate of reduced copper. The percentage conversion of potassium gluconate to potassium 2-ketogluconate was then estimated by comparison with previously prepared standards made with weighed quantities of calcium 2-ketogluconate. The conversion was recorded as 75, 50 or 25 %, or none. Exact quantitative relationships were deemed unessential to establish the capability of these organisms to oxidize potassium gluconate to potassium 2-ketogluconate.

Slime production

The same medium used for determining the ability of these organisms to oxidize potassium gluconate was used for slime production. (Slime production as a significant diagnostic characteristic of *P. aeruginosa* was first suspected when an associate, Dr H. J. Koepsell, detected its presence in a flask which had been allowed to stand for several hours.)

On the completion of observations for oxidation of potassium gluconate, the flasks were allowed to stand at room temperature until maximum slime formation occurred. This was usually 4 days after the flasks were removed from the shaker. Maximum slime formation was recognized when all of the following phenomena, listed in the order of appearance, were observable.

Slimy pellicle. The pellicle which forms in still culture appears slimy when the flask is gently swirled. This pellicle usually appears after overnight incubation in still culture.

Reverse swirl. When a flask containing slime is vigorously swirled and then set on a table, the swirling liquid rapidly slows to a stop and then abruptly reverses direction. This phenomenon is usually weak after overnight incubation, but rapidly becomes stronger until about the fourth day. Thereafter, in some strains this phenomenon increases in intensity, although more slowly, until the liquid becomes quite viscous. With such strains no diminution has been observed, even after several weeks. In other strains, however, a maximum is reached, after which the sliminess subsides until it can scarcely be detected.

Transitory film formation. After the pellicle has been submerged by vigorous swirling, and while it is still being gently swirled, examination of the flask by transmitted light reveals a slimy, transparent film on the walls of the flask where the liquid has splashed. This film slowly flows down into the body of the liquid again.

'Oyster' formation. After film formation and the reverse swirl phenomenon have become strong there usually is a differentiation of a part of the slime into an 'oyster-like mass' which becomes visible when the centrifugal action of swirling throws the remainder of the fluid towards the walls of the flask, leaving the '*oyster*' at the apex of the vortex.

When slime formation has progressed to the stage where there is a good reverse swirl, a wire hook or loop inserted into the liquid and withdrawn draws the slime out into a thread which is quite thick and viscid, and in some cases may be stretched a foot or more before it breaks.

Strains of bacteria observed during this study included all strains received as *P. aeruginosa*, strains re-identified by the writer as *P. aeruginosa*, and finally a few strains isolated at this Laboratory and identified as *P. aeruginosa*. The sources of these strains are shown in Table 1.

RESULTS

Pyocyanine production

Thirty-six strains produced pyocyanine in Gessard's glycerol peptone agar stabs and in Burton's medium. It must be pointed out, however, that strain NRRL-B-219, although it usually produces pyocyanine in either medium, has failed to do so on at least one occasion in each. Another strain, NRRL-B-255, has always produced pyocyanine in Burton's medium, but failed to do so on one occasion in a Gessard stab.

Nine strains have been consistently negative in regard to pyocyanine production in both media. On the other hand, three strains which have never given a positive test for pyocyanine in Gessard's medium have done so in Burton's medium, two of these producing but a trace. Likewise, four strains consistently positive in Gessard stabs have produced but a trace of pyocyanine (three strains) or none (one strain) in the medium of Burton *et al.* Thus these media are equally effective in detecting pyocyanogenic strains. However, neither can be depended upon to reveal all of them, nor can a single negative result be accepted as reliable.

It is suggested that Burton's medium be used for routine surveys and that strains which fail at the initial test to produce pyocyanine be re-checked in the same medium and also in Gessard stabs. Burton's medium is selected since its liquid form makes it more easily prepared and dispensed and it is possible to test samples at intervals during an observation period of 10 days to 2 weeks. A summary of results obtained with each medium for each strain is shown in Table 1.

Temperature relationships

Of 138 pseudomonads tested for growth on TGY slants in the 41° ± 1 water-bath, 57 grew well. In addition to these, 43 others grew at 36° ± 1, while all 138 grew well at 30°. Among the 57 which grew well at 41° ± 1 were the 43 pyocyanogenic strains of *P. aeruginosa* and the 9 apyocyanogenic strains identified as members of the same species on the basis of the three other characteristics shown herein to be diagnostic for it. These data are given in

Table 1. *Strains studied and pyocyanogenic capacity of each in two media*

NRRL no.	Received as	Pyocyanine production in		Donor
		Gessard stabs	Burton's medium	
B-7	*Pseudomonas fluorescens*	–	–	N. R. Smith, as no. 112
B-12	*P. fluorescens*	–	–	ATCC, as no. 142
B-23	*P. vendrelli*	+++	+++	ATCC, as no. 7700
B-26	*P. aeruginosa*	++	++	N. R. Smith, as no. 110
B-27	*P. aeruginosa*	+++	+++	N. R. Smith, as no. 111
B-79	*P. synxantha*	++	+++	H. F. Long
B-189	*P. fluorescens*	++	+++	E. McCoy
B-211	*P. aeruginosa*	++	++	NRRL isolate
B-217	*Pseudomonas* sp.	++	+++	L. D. Bushnell, as Bear
B-218	*Pseudomonas* sp.	++	+++	L. D. Bushnell, as no. 82
B-219	*Pseudomonas* sp.	++	++	L. D. Bushnell, as K-4
B-220	*Pseudomonas* sp.	++	++	L. D. Bushnell, as PS L
B-221	*Pseudomonas* sp.	–	–	L. D. Bushnell, as Ps
B-222	*Pseudomonas* sp.	+	++	L. D. Bushnell, as YS-6
B-241	*P. aeruginosa*	–	–	ATCC, as no. 257
B-247	*P. aeruginosa*	–	–	ATCC, as no. 97
B-248	*P. aeruginosa*	–	–	ATCC, as no. 256
B-249	*P. aeruginosa*	–	–	ATCC, as no. 260
B-250	*P. aeruginosa*	–	–	ATCC, as no. 262
B-255	*P. aeruginosa*	++	++	K. H. Lewis, as A-4
B-256	*P. aeruginosa*	++	+++	K. H. Lewis, as D-1
B-257	*P. aeruginosa*	+++	++	K. H. Lewis, as A-3
B-264	*Pseudomonas* sp.	+++	+++	C. N. Stark, as no. 1
B-265	*Pseudomonas* sp.	++	+++	C. N. Stark, as no. 2
B-275	*Pseudomonas* sp.	–	–	C. N. Stark, as no. 150
B-323	*P. fluorescens*	+	+	M. Solowey, as no. 4089
B-325	*P. aeruginosa*	++	+++	NRRL isolate
B-428	*P. fluorescens*	++	++	M. Solowey, as no. 6918–1
B-431	*P. fluorescens*	+++	+++	M. Solowey, as no. 4215
B-450	*P. aeruginosa*	+	++	NRRL isolate
B-451	*P. aeruginosa*	+	+++	NRRL isolate
B-452	*P. aeruginosa*	+++	+++	NRRL isolate
B-534	*P. aeruginosa*	++	+++	NRRL isolate
B-716	*P. aeruginosa*	+	++	NRRL isolate
B-743	*P. chlororaphis*	+++	+++	R. Y. Stanier, as A.3.30
B-771	*P. aeruginosa*	++	+++	ATCC, as no. 10145
B-786	*P. syncyanea*	++	++	P. H. H. Gray
B-800	*P. aeruginosa*	+++	+++	ATCC, as no. 9027
B-853*	*P. polycolor*	+++	++	W. H. Burkholder, as PP2
B-903†	*P. aeruginosa*	++	++	E. W. Schultz, as U-21*d*
B-904†	*P. aeruginosa*	++	±	E. W. Schultz, as U-21*e*
B-937	*P. chlororaphis*	+++	+++	M. P. Starr, as MD 44.1
B-982*	*P. marginalis*	+++	++	NCTC, as no. 1995
B-996	*P. pyocyanea* var. *erythrogenes*	–	±	NCTC, as no. 5083
B-997	*P. pyocyanea* var. *erythrogenes*	++	–	NCTC, as no. 6749
B-1000‡	*P. aeruginosa*	–	++	E. J. Hehre, as 'Henriksen'
B-1019‡	*P. aeruginosa*	++	±	L. H. Schwarz, as LS-1
B-1069	*Malleomyces haemorrulcogenes*	–	±	NCTC, as no. 8028
B-1080	*Pseudomonas pyocyanea* var. *erythrogenes*	++	++	NCTC, as no. 4844
B-1083	*P. aeruginosa*	++	+++	NRRL isolate
B-1087	*P. aeruginosa*	+	++	NRRL isolate
B-1088‡	*P. aeruginosa*	++	±	ATCC, no. 10197

* Presumably phytopathogens. The strain of *P. polycolor* and one other, previously has been shown by Elrod & Braun (1942) to be the same as *P. aeruginosa*. Mehta & Berridge (1924) and Elrod & Braun (1942) believe *P. marginalis* is the same as *P. aeruginosa*. St John-Brooks, Nain & Rhodes (1925) confirm this relationship so far as this particular strain is concerned, but not with another strain received from Erwin F. Smith.

† Strains B-903 and B-904 are two strains of E. W. Schultz's gelatinous variant (1947).

‡ Strains B-1019 and B-1088 are strains of the mucoid *P. aeruginosa* described by Schwarz & Lazarus (1947). B-1000 is a strain of the mucoid *P. aeruginosa* described by Henriksen (1948).

ATCC = American Type Culture Collection. NCTC = National Collection of Type Cultures (Great Britain). NRRL = Northern Regional Research Laboratory (U.S.A.).

Table 2. The remaining five strains which grew at 41° ± 1 failed to meet the other requirements for inclusion in the species.

Table 2. *Comparison of selected growth temperatures with potassium gluconate oxidizing capacity of some pseudomonads*

Grow well at	No. of strains	No. of strains converting gluconate to 2-ketogluconate to extent of		
		75 %	50 %	25 % or less
41° ± 1	57	52	0	5
36° ± 1	43	30	4	9
30°	38	26	0	12
Total	138	108	4	26

Thus, this investigation confirms reports that *P. aeruginosa*, whether or not pyocyanogenic, grows well at 42°. Like other investigators, I have encountered strains able to grow at this temperature which are not *P. aeruginosa*. Growth of *P. aeruginosa* at 45° is poor, when it occurs.

Oxidation of potassium gluconate

Lockwood, Tabenkin & Ward (1941) reported the production from glucose of 2-ketogluconic acid by 22 strains of *Pseudomonas* representing 12 species. Five strains of *P. aeruginosa* were among the 22 strains investigated, including *P. vendrelli*, which subsequently was shown to be *P. aeruginosa* (Tobie, 1946); and two strains listed by Lockwood *et al.* as *P. fluorescens* (ATCC-142 and the N.R. Smith strain), which have been currently re-examined and are now identified as apyocyanogenic strains of *P. aeruginosa*. Their observation points to the possibility that the entire genus may be characterized on the basis of an oxidative attack on glucose and other carbon sources. With a view to elucidating the value of this property in the systematic relationships of these and related organisms, a survey of several hundred strains representing many species was undertaken. Results of the survey will be published later. During the survey it was found that all strains of *P. aeruginosa* oxidized glucose to a compound which was shown to be 2-ketogluconic acid. The five strains of *P. aeruginosa* included in the report by Lockwood *et al.* (1941) are among the strains herein reported.

Working exclusively with *P. aeruginosa*, ATCC-9027 (NRRL-B-800) Norris & Campbell (1949) identified 2-ketogluconic acid as an intermediate in the oxidation of glucose, and also demonstrated that the resting cells were able to oxidize gluconate. The strain with which they worked is included among the strains studied in this investigation. Stubbs, Lockwood, Roe, Tabenkin & Ward (1940) demonstrated the ability of an organism later identified as a pseudomonad to produce 2-ketogluconic acid from calcium gluconate.

Inasmuch as gluconic acid does not reduce Benedict's reagent and yet may be oxidized to 2-ketogluconic acid which does reduce it, the advantage of gluconic acid as the carbon source in studying the oxidative metabolism of these organisms is obvious. The potassium salt of gluconic acid was selected for use in our medium because it gives clear solutions and is readily available. It was furnished at a concentration of 4 % (w/v), at which level potassium 2-ketogluconate accumulated and could be estimated at convenient intervals.

All pyocyanogenic strains of *P. aeruginosa* rapidly oxidized potassium gluconate to a reducing compound, presumably potassium 2-ketogluconate, and at the end of 2 days had effected a conversion of at least 50 % of the gluconate to 2-ketogluconate. By the fourth day the conversion usually was 75 % or more. Shaking was continued until a conversion equivalent to at least 75 % was obtained at two successive observations. This had usually been accomplished by the seventh day. The nine apyocyanogenic strains, tested in like manner, produced similar results.

The fact that many strains, other than those identifiable as *P. aeruginosa*, oxidized gluconate to 2-ketogluconate does not invalidate use of this characteristic for identification of the species; as shown in Table 2, only 52 strains of 112 able to oxidize gluconate were also able to grow well at 41° ± 1. Those 52 strains are *P. aeruginosa* on the basis of this and two other characters found to be characteristic of the species.

Slime formation in potassium gluconate broth

From time to time reports have appeared in the literature of the isolation of mucoid variants (Schwarz & Lazarus, 1947; Henriksen, 1948; Sonnenschein, 1928; Dahr & Kolb, 1936) and of gelatinous variants (Schultz, 1947) of *P. aeruginosa.* Neither type of growth is common, such forms being regarded as more or less unstable variants of the usual butyrous type. However, it was possible to provoke, in all strains of *P. aeruginosa* tested, a style of growth which is best described as 'slimy'. This type of growth develops in a tryptone yeast-extract K_2HPO_4 basal medium containing 4 % potassium gluconate. When glucose is substituted for the potassium gluconate, slime formation does not occur. (Calcium gluconate was not able to replace potassium gluconate as the carbon source.) The optimum temperature for slime production is 36°, although slime forms also at 30° and 25°, but more slowly. At 41° there is rapid production of slime, but this never becomes as well developed as at 36°. Phosphate ion appears to be essential for maximum development of the slimy material; yeast extract is not essential and may be excluded when the tryptone is increased to at least 0·25 % (w/v).

In shake flasks slime is seldom observed. It is easily recognized, however, when flasks that have been shaken for 3 or 4 days are allowed to stand for an additional 18-24 hr.

In static culture at 36° (water-bath temperature), which represents optimum conditions, sliminess may be observed after 18–24 hr. As in shake flasks which are allowed to stand, the degree of sliminess increases until about the fourth day, but with no appreciable change thereafter.

Slime formation in this medium may also be observed in test-tubes incubated at 36°. The various manifestations of slime are not so readily seen under these circumstances, but the reverse swirl effect can be demonstrated.

On agar slants, prepared by adding 1·5 % of agar to the medium used in liquid culture, sliminess developed only in the tubes inoculated with a gelatinous variant received from E. W. Schultz (NRRL-B-904), and with a mucoid strain (NRRL-B-1019) sent by L. H. Schwarz. The other gelatinous strain and the other mucoid strains were not tested. All other strains tested produced a growth which may be described as 'elastic'.

Several hundred strains belonging to many genera in the family Pseudomonadaceae, primarily in the genus *Pseudomonas*, have been inspected for slime formation. Although the degree of sliminess observed has never approached that attained by the *P. aeruginosa* cultures, a few have developed a relatively low degree of sliminess. This fact does not invalidate the criteria herein offered for the identification of *P. aeruginosa*, since the identification is not dependent upon slime formation alone but upon two additional characteristics which correlate with it, as shown in Table 3. None of the slime-forming organisms other than *P. aeruginosa* were able also to grow at 41° and to oxidize potassium gluconate to a reducing compound.

Table 3. *Comparison of selected growth temperatures with ability to produce slime in potassium gluconate broth*

Grow well at	No. of strains	No. of strains showing intensity of slime formation as		
		Strong	Moderate	Weak
41° ± 1	53	52	0	1
36° ± 1	16	0	0	16
30°	17	2	2	13
Total	86	54	2	30

The 52 strains which grew at 41° ± 1 and produced profuse sliminess were the 43 pyocyanogenic and 9 apyocyanogenic strains of *P. aeruginosa*. The remaining strain which grew at 41° ± 1 did not approach *P. aeruginosa* in slime-forming ability and, more important, did not oxidize gluconate to 2-ketogluconate.

DISCUSSION

It is believed that the three correlating characteristics presented here will make it possible to remove from the '*Pseudomonas fluorescens* species group' those organisms which are truly *P. aeruginosa* and perhaps thereby diminish confusion to the point where further progress can be made in establishing the true relationships of the remaining members of the group.

The ability of *P. aeruginosa* to oxidize potassium gluconate to 2-ketogluconate was implied by the work of Norris & Campbell (1949) and Stubbs *et al.* (1940). It has been confirmed in the present investigation and shown to be of taxonomic value when used as a diagnostic characteristic along with ability to grow at 42° and to produce slime from potassium gluconate.

Slimy or mucoid strains of *P. aeruginosa* are seldom encountered, and have usually been associated with pathogenic conditions in man. Upon the potassium gluconate medium used in this investigation we have found that all strains of

P. aeruginosa grow in the mucoid form. These included pyocyanogenic and apyocyanogenic strains, gelatinous variants, mucoid strains, and the usual types which produce butyrous growth on solid media. Slime production is a constant characteristic, showing correlation with two other constant characteristics, and is believed to be, in company with them, a reliable characteristic for use in identification of *P. aeruginosa.*

In 1896, Pottien described *Bacillus fluorescens capsulatus*, a mucoid organism isolated from intestinal contents in several fatal cases of cholera nostras. It has been suspected that this was another instance where the investigator was dealing with a mucoid strain of *Pseudomonas aeruginosa.* However, according to the original description, such is definitely not the case, since *Bacillus fluorescens capsulatus* fermented glucose and glycerol with the evolution of gas and produced an unidentified crystalline compound in several media. Gas production in glycerol media relegates Pottien's species to the acid- and gas-producing group (*Aeromonas*).

The value of more precise means of identifying *Pseudomonas aeruginosa* is illustrated by the following occurrence. Dr Aldo Castellani isolated a microorganism from a pathogenic condition of human beings and assigned it the name *Malleomyces haemorrulcogenes.* He sent cultures to S. T. Cowan (National Collection of Type Cultures, Colindale, London) who reidentified it as *Pseudomonas pyocyanea* (*P. aeruginosa*). A culture was forwarded to me and I have confirmed Dr Cowan's opinion by the methods presented in this report. In the meantime, Castellani (1950) has reassigned this organism to the genus *Pseudomonas*, but has retained the specific epithet *haemorrulcogenes.*

The value of means for the identification of *P. aeruginosa* supplementary to pyocyanine formation is demonstrated further by the fact that 11 strains received as members of five other species of the genus were identified as *P. aeruginosa.* Nine of them produced pyocyanine in Gessard's medium and in Burton's medium, while two failed to do so in any medium tried. It is apparent that the certain identification of *P. aeruginosa* requires the use of media and conditions specifically designed and proved most suitable to elicit characteristic responses. Failure to do so is bound to result in misnaming of many isolates.

Strain NRRL-B-853, received as *P. polycolor* is one of two strains shown by Elrod & Braun (1942) to be pyocyanogenic and identical with *P. aeruginosa.* We have confirmed this relationship by means of three additional characters found to be characteristic of the latter.

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The Catabolic Activity of Washed Suspensions of *Haemophilus pertussis*

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SUMMARY: Washed suspensions of a laboratory strain of *Haemophilus pertussis* did not take up oxygen with, or ferment, five carbohydrates, nor did the same strain produce detectable acid from fourteen carbohydrates during growth. Of twenty-one amino-acids tested only proline, serine, alanine, aspartic and glutamic acids were oxidized by washed suspensions at an appreciable rate. The first four of these were oxidized to completion but with glutamic acid only 80 % of the theoretical uptake of oxygen occurred; however, in the presence of dinitrophenol it, too, was oxidized to completion. Cells oxidizing glutamic acid in the presence of arsenite produced α-ketoglutaric acid.

During the logarithmic growth of *H. pertussis* glutamic acid disappeared from Cohen & Wheeler's medium. The organism grew in a Cohen & Wheeler's medium in which the Casamino acids had been replaced by glutamic acid. These results suggest that glutamic acid can serve as energy, carbon and nitrogen source for *H. pertussis*.

Some of the findings were confirmed with freshly isolated strains.

Haemophilus pertussis was included in the genus *Haemophilus* by Winslow, Broadhurst, Buchanan, Krumwiede, Rogers & Smith (1920) because of a supposed dependence for growth on blood, or other body fluids. Later work has disproved this and tends to separate *H. pertussis* from other members of the genus. *H. pertussis* does not require *X* or *V* factors (Rivers, 1922*a*, *b*; Fildes, 1923). Hornibrook (1939) grew it in a mixture of casein hydrolysate, an organic sulphur compound (cystine or glutathione), inorganic salts and starch. Later (Hornibrook, 1940) he simplified the medium to a defined mixture of amino-acids, salts, starch and nicotinic acid. Using a modification of Hornibrook's medium, Farrell & Taylor (1945) noted that some strains required thiamine as well as nicotinic acid and others would not grow without liver extract. Pollock (1949) also noted that defined media would not support the growth of small inocula without yeast extract.

Dawson, Farnworth, McLeod & Nicholson (1951) found that of a number of amino-acids added to Bordet & Gengou's medium, aspartic and glutamic acids tended to stimulate growth and serine to inhibit it.

Pollock (1947), using a trained strain, traced the function of blood in media for *H. pertussis* to the ability of serum albumin to bind traces of unsaturated fatty acids, which are toxic to the organism. He found that starch and charcoal, which also bind unsaturated fatty acids, could replace serum albumin. Further, he showed (Pollock, 1949) that his strain actually produced toxic fatty acids during growth in defined media.

Little is known of the metabolic activities of *H. pertussis*, but the following facts appear significant:

(i) Its growth is favoured by adequate aeration (Bordet & Gengou, 1907;

Hornibrook, 1939; Wilson, 1945; Farrell & Taylor, 1945; Cohen & Wheeler, 1946; and Verwey, Thiele, Sage & Schuchardt, 1949), though Ungar, James, Muggleton, Pegler & Tomich (1950) claim that agitation depresses the growth of virulent strains.

(ii) It does not produce acid from sugars (Stillman & Bourn, 1920; Rivers, 1922*b*; Lawson, 1939).

(iii) Various workers report that the medium becomes alkaline during growth, suggesting a vigorous metabolism of amino-acids, and Ungar *et al.* (1950) state that during 15 days' growth *H. pertussis* completely removed from the medium glutamic and aspartic acids, serine, glycine and proline.

It seems probable, therefore, that *H. pertussis* obtains its energy for growth by aerobic metabolism of amino-acids. The oxidative metabolism of the organism has been studied in an attempt to determine its principal sources of energy and carbon as a preliminary to further investigations of its nutrition and the role of unsaturated fatty acids.

METHODS

Organisms. Strains 318, 723, 724 and 1437 were isolated in this laboratory and had been kept dried for 2 years prior to use. Strain 2916 was kindly supplied by Mr A. F. B. Standfast. A cellar of dried cultures was prepared by Stamp's (1947) method, and a fresh tube was opened monthly and maintained on Bordet & Gengou's medium. Periodic tests showed that the strains were always agglutinated by Phase I serum (from the Standards Laboratory) at a dilution of 1/16,000 and frequently by 1/32,000. Towards the end of the investigation a virulence test on strain 724 was performed by Mr A. F. B. Standfast, of The Lister Institute of Preventive Medicine, Elstree. He found that the LD 50 on the 28th day, for mice inoculated intranasally, was $3{\cdot}6 \times 10^6$, i.e. group B (Standfast, 1951) and stated that the strain was of moderate to high virulence.

Some of the findings were confirmed with fresh strains.

Media. Bordet & Gengou's medium was prepared according to the method given by Mackie & McCartney (1948).

Two liquid media were used. Most of the work was done with Cohen & Wheeler's (1946) medium prepared as described by them, but Distillers Co. Ltd. pressed yeast was used to prepare the dialysate. Difco Casamino acids were obtained from Messrs Baird and Tatlock Ltd. The medium was distributed in 100 ml. lots in 500 ml. Erlenmeyer flasks. Some of the earlier experiments were done with a modification (designated medium VI) containing an acid hydrolysate of casein (Gladstone & Fildes, 1940) in place of Casamino acids. The medium was distributed in Roux bottles.

Washed suspensions. Inocula for the flasks were prepared by scraping growth off Bordet & Gengou's medium and suspending it in medium to a standard opacity. Plate counts were occasionally performed and showed that the inoculum used contained approximately 10^9 viable cells per flask. Roux bottles of medium VI were grown for 3 days, and flasks of Cohen & Wheeler's medium were incubated on a mechanical shaker for 22 hr., both at 35°. The cells were centrifuged from the medium, washed twice with distilled water and resuspended in water. Using a Hilger 'Biochem' absorptiometer a calibration curve was constructed relating extinction to the dry weight of cells. Because of the marked decrease in respiration when they were washed, cells from medium VI were suspended in 5 % yeast dialysate prepared by the method described by Cohen & Wheeler (1946).

Ammonia determinations. Samples were acidified at the time of collection and subsequently distilled with soda in the apparatus described by Markham (1942). A few drops of tri-amyl citrate were added to prevent frothing. The ammonia was distilled into 2 % (w/v) boric acid and titrated with 0·01 N hydrochloric acid. In some experiments the ammonia was estimated with Nessler's reagent (Johnson, 1941).

Gas exchanges were measured by conventional Warburg methods as described by Umbreit, Burris & Stauffer (1945). Except where otherwise stated, each manometer flask contained: cell suspension, 1·0 ml.; 0·2 M phosphate buffer (pH 7·6), 0·5 ml.; substrate, 0·2 ml.; water, 0·3 ml.; and 10 % potash, 0·1 ml., in the centre well. The flasks were shaken at 120 osc./min. with a 3 cm. stroke in a bath at 37°.

Glutamate estimations were performed by Gale's (1945) method as modified by Krebs (1948).

Substrates. We are indebted to various members of the University Biochemistry Department for samples of D-glutamic acid, L-serine and sodium pyruvate. The other amino-acids used were commercial preparations obtained from British Drug Houses Ltd., Roche Products Ltd. and L. Light & Co. Ltd.; sodium lactate was prepared from 'Analar' lactic acid; α-ketoglutaric and oxaloacetic acids were obtained from Light & Co. Ltd.

RESULTS

Fermentation of carbohydrates during growth

Five strains were inoculated into 10 ml. lots of medium VI containing 1 % (w/v) of glucose, fructose and maltose in 100 ml. conical flasks. The flasks were then incubated at 35° without shaking for 5 days, and the pH of each flask was measured. In every case the pH had risen from the initial value of 7·2 to 8·0–8·4. In another experiment strain 724 was inoculated into 10 ml. lots of Cohen & Wheeler's medium containing 1 % (w/v) of the sugar to be tested in 100 ml. conical flasks and in $6 \times \frac{3}{4}$ in. tubes. The initial pH was 6·8–7·2, and after 5 days' incubation at 35° without shaking it had risen, in every case, to 8·0–8·4. After a further 5 days there was no change. The 'sugars' tested in this experiment were glucose, fructose, galactose, lactose, sucrose, glycerol, adonitol, arabinose, rhamnose, mannitol, dulcitol, sorbitol, maltose and raffinose.

Oxidation and fermentation of carbohydrates by washed suspensions

Cells were grown on Cohen & Wheeler's medium, washed suspensions prepared from them as previously described and the oxygen uptake with glucose, maltose, sucrose, lactose and starch was measured in Warburg flasks. The anaerobic production of carbon dioxide from these five carbohydrates was also measured in Warburg flasks. The oxygen uptake and carbon dioxide output with these five substances were not materially different from the blank rates. From this experiment, combined with the preceding results during growth, it would appear that, even if these carbohydrates are attacked, they are unlikely to provide the main source of energy for growth.

Production of ammonia during growth

Preliminary experiments showed that the rise of pH of the medium during growth of *H. pertussis* was accompanied by the production of ammonia. Flasks (250 ml. capacity) containing 50 ml. of medium VI were inoculated with five strains of *H. pertussis*. The flasks were incubated unshaken at 35°, and samples were removed daily for estimation of turbidity and for ammonia determination. All the strains increased the ammonia content of the medium by about 5μmol./ml. in 4 days, the rise being roughly parallel with the growth.

Oxidation of amino-acids

As *H. pertussis* grows best in well-aerated culture it seemed justifiable to investigate the respiration of resting suspensions of organisms and to see which amino-acids, if any, were oxidized by washed cells. Cells were grown in medium VI, washed twice with water, resuspended in 5 % yeast dialysate, and put up to oxidize the amino-acids which Ungar *et al.* (1950) found to be removed from the medium. The results are shown in Table 1. With the exception of glycine, all the substrates increased the oxygen consumption and ammonia production. The uptake with medium VI was equal to that with glutamic acid during the first hour, but thereafter it fell off, which suggests that the oxidation of the medium may be largely due to its glutamic acid content.

Table 1. *Oxidation of five amino-acids by strain* 724

Substrate ...	Nil	Medium VI	L-Glutamic acid	DL-Aspartic acid	Glycine	DL-Serine	DL-Proline
	Oxygen uptake (μl.)						
Time (min.)							
60	56	208	212	74	40	76	110
120	73	307	462	118	61	121	187
180	82	357	721	148	71	146	236
210	86	374	852	156	76	157	280
μmol. ammonia produced in 210 min. (blanks subtracted)	1·0	3·5	10·0	3·0	1·0	3·8	2·5

Cells from medium VI made up in 5 % yeast dialysate to contain 5·4 mg./ml.

Oxygen uptake with all the other amino-acids that were available was then investigated. With washed suspensions the following amino-acids did not give oxygen uptakes appreciably higher than the blank: DL-valine, L-tryptophan, L-arginine, L-lysine, L-hydroxyproline, DL-leucine, DL-isoleucine, DL-norleucine, DL-methionine, DL-threonine, DL-norvaline, DL-phenylalanine, L-histidine, β-alanine and L-tyrosine. With D-glutamic acid and DL-α-alanine the Q_{O_2} was approximately one-seventh of that with L-glutamic acid.

To test whether incorporating extra amino-acid in the growth medium would increase the rate of oxidation by washed cells proline, glycine, alanine and aspartic acid were added to four lots of Cohen & Wheeler's medium to give a final concentration of 0·025 M and strain 724 was grown in these media. The rate of oxidation of proline by proline-grown cells was 25 % higher than by cells out of the ordinary medium. The rate of oxidation of the other three acids was not increased by growth in the fortified media.

The effect of varying the period of growth before harvesting the cells was investigated by inoculating several flasks of Cohen & Wheeler's medium with strain 724 and preparing washed suspensions after 12, 18, 23 and 39 hr. growth. The rates of oxidation of glutamic acid, proline, serine and alanine, did not alter greatly between 12 and 23 hr., but by 39 hr. they had all declined to varying degrees. With aspartic acid, the cells showed little activity and this declined steadily from 12 to 39 hr. The results were confirmed with cells grown in 800 ml. medium in a Winchester bottle from which samples were removed for turbidity readings and the preparation of washed suspensions. As the growth was logarithmic up to 23 hr. the enzymes oxidizing these five amino-acids were most active in growing cells. The slow oxidation of aspartic acid will be discussed more fully later.

As the work had so far all been done with strain 724, the respiration of four other dried strains and five freshly isolated strains was investigated to see whether strain 724 was typical. The results given in Table 2 show that strain 724 behaved in the same way towards the substrates as the other strains examined.

Detailed investigation of amino-acid metabolism

Effect of pH *on the oxidation of glutamic acid.* Strain 724 was grown in Cohen & Wheeler's medium, suspended in distilled water and put into Warburg flasks over a pH range from 8·2 to 4·5. The activity in phosphate buffer varied little between pH 8·2 and 6·9. Cells in borate buffer at pH 8·0 were much less active than cells in phosphate buffer; higher pH values were not investigated.

The effect of yeast dialysate on glutamic acid oxidation. Cells grown in medium VI lost a large part of their activity during washing and this activity was restored by adding yeast dialysate to the manometer flasks. Even after subtracting the increased blank some 50 % stimulation of the respiration in glutamic acid remained. The yeast dialysate could not be replaced by a mixture of B vitamins, including biotin and pyridoxal phosphate. Cells grown in Cohen & Wheeler's medium did not show the stimulation by yeast dialysate, nor suffered any reduction in their activity by dilution of the cell suspension The matter was not pursued further at this point.

The relation between glutamic acid oxidized and the amount of oxygen taken up. The amount of oxygen taken up per mol. glutamic acid consumed was next

Table 2. *The oxidation of various substrates by washed suspensions of five dried and five freshly isolated strains of* Haemophilus pertussis

		Q_{O_2} with various substrates										
Strain	Days*	Nil	L-Glutamic acid	DL-Aspartic acid	DL-Alanine	DL-Serine	DL-Proline	Lactate	Glucose	Starch	Maltose	Lactose
318	Dried	1·5	45·0	1·5	5·9	2·9	3·7	.	.	.	.	.
723	Dried	2·1	58·0	2·2	6·0	3·6	5·3	.	.	.	.	.
1437	Dried	2·2	77·0	1·7	7·2	3·8	7·6	.	.	.	.	.
2916	Dried	2·3	61·0	1·2	6·7	4·2	5·7	.	.	.	.	.
724	Dried	1·8	71·0	3·3	6·0	4·5	8·0	.	.	.	.	.
352	8	0·5	38·3	0·8	.	.	5·5	44·0	1·1	.	1·7	2·1
351	10	1·0	26·0	0·65	.	.	3·0	31·0	1·25	.	0·9	0·4
926	10	3·2	46·0	7·3	.	14·7	34·0	26·5	4·3	4·8	3·65	.
1385	10	7·1	65·0	14·4	19·5	16·3	26·3	36·0	7·1	7·3	7·5	.
1903	11	1·0	70·6	6·4	13·8	7·0	9·1	56·0	1·3	1·3	1·6	.

* Time between the taking of the swab and the performance of the experiment.
All suspensions contained 5 gm. cells/ml.

Table 3. *Oxidation of glutamic acid to completion by strain 724 with and without 2:4-dinitrophenol*

								mol./mol. glutamic acid		
Exp. no.	Cells grown in	Suspension strength (mg./ml.)	D.N.P. 0·00075 M (final)	Substrate (μmol.)	Oxygen uptake (μmol.)	Carbon dioxide output (μmol.)	Ammonia produced (μmol.)	O_2	CO_2	NH_3
1	Medium VI	5·0	–	10·0	30·5	.	.	3·05	.	.
2	Cohen & Wheeler	4·5	–	4·0	12·8	15·1	3·35	3·20	3·75	0·83
3	Cohen & Wheeler	4·5	–	4·0	13·7	16·1	3·1	3·42	4·02	0·77
4	Medium VI	17·7	+	10·0	41·0	.	.	4·1	.	.
5	Cohen & Wheeler	23·5	+	4·0	15·6	18·5	3·95	3·90	4·60	0·98
6	Cohen & Wheeler	24·0	+	4·0	17·5	19·4	4·00	4·35	4·85	1·00

investigated. Klein (1940) concluded that *H. parainfluenzae* oxidized glutamic acid thus:

$$C_5H_9NO_4+2{\cdot}5O_2=CH_3COOH+H_2O+3CO_2+NH_3.$$

Complete oxidation of glutamic acid would require 4·5 mol. of oxygen per mol. glutamic acid, thus:

$$C_5H_9NO_4+4{\cdot}5O_2=3H_2O+5CO_2+NH_3.$$

Using 10 μmol. glutamic acid in each flask it was shown that rather over 3 mol. oxygen were taken up per mol. glutamic acid (Table 3, Exp. 1). The yield of carbon dioxide and ammonia were then measured in parallel with the oxygen uptake (Table 3, Exps. 2 and 3). As 20 % of the carbon was not accounted for, an attempt was made to detect volatile acids at the end of glutamic acid oxidation.

Strain 724 was grown in Cohen & Wheeler's medium and the washed cells were put up in eight manometer flasks, each containing 10 μmol. glutamic acid. A blank reaction mixture equal to the contents of the eight flasks was put up in a 100 ml. conical flask on a shaker in the incubator. When the respiration had ceased the contents of the manometer flasks were pooled, the flask contents and the blank reaction mixture were acidified with 2 ml. 4 N sulphuric acid and 2 ml. 20 % (w/v) sodium tungstate added. After filtration and washing of the precipitate, the combined filtrates and washings were steam-distilled in the Markham apparatus. The distillate (150 ml.) was collected and titrated with 0·02 N soda in a CO_2-free atmosphere. In a series of four experiments a mean value of 0·035 equivalent of acid were recovered for each mol. of glutamic acid oxidized. As this quantity was not considered to be significant and the discrepancies between the observed and calculated figures for oxygen, carbon dioxide and ammonia were too great to be ascribed to experimental error, it seemed probable that oxidative assimilation was occurring and the possibility of inhibiting this was considered.

2:4-Dinitrophenol, widely used for uncoupling assimilation from oxidation (Clifton, 1946), did in fact increase the total oxygen taken up per mol. glutamic acid consumed. As it also decreased the rate of respiration a stronger cell suspension was used. Table 3, Exp. 4, shows that in the presence of 2:4-dinitrophenol 4·1 mol. oxygen were taken up for each mol. glutamic acid used. Table 3, Exps. 5 and 6, shows the oxygen uptake and production of carbon dioxide and ammonia from glutamic acid by cells of *H. pertussis* poisoned with 2:4-dinitrophenol. The observed ratios compared favourably with the theoretical ratios for complete oxidation.

The course of the reaction. Having shown that *H. pertussis* oxidized glutamic acid completely, it was of interest to see what possible intermediates were oxidized at a similar rate, in particular the substances postulated by Klein as intermediates in the oxidation of glutamic acid by *H. parainfluenzae*. A washed suspension of strain 724 was put up in a Warburg respirometer to oxidize all the available acids with one to five carbon atoms. Lactate and succinate were initially attacked as rapidly as glutamic acid. α-Ketoglutarate was oxidized at about 50 % of the rate with glutamate. With oxaloacetate, fumarate, malate, propionate, acetate and formate the oxygen uptake did not exceed that of the blank. The oxygen uptake with pyruvate was about 15 % of that with glutamate. The three keto-acids were put up anaerobically with cells suspended in bicarbonate buffer and the carbon dioxide output measured. Only with α-ketoglutaric acid did the output exceed that of the blank.

α-Ketoglutaric acid is known to be produced from glutamic acid by animal tissue (Krebs, 1933), yeast (Euler, Adler & Eriksen, 1937) and *Bacterium coli* (Adler, Hellström, Gunther & Euler 1938). This reaction was looked for with *H. pertussis*. By using inhibitors which have been successfully employed for blocking the oxidation of keto-acids (toluene, 2:4-dinitrophenylhydrazine, nitrite and cyanide), an attempt was made to obtain a quantitive uptake of half a mol. of oxygen. These substances either completely stopped respiration or decreased the respiration in glutamate almost to the level of the blank. Arsenious oxide was more satisfactory. Cell suspensions containing 19 mg. dry weight were put into Warburg flasks to oxidize 20 μmol. L-glutamic acid in the presence of various concentrations of arsenious oxide. The manometers were read for 240 min., taken down and the ammonia estimated. In the absence of inhibitor the oxygen/ammonia ratio in mol. was 3·0:1·0, suggesting that assimilation of the product of deamination was occurring. With 0·001 M arsenious oxide assimilation appeared to be inhibited and the ratio approached the theoretical value for complete oxidation, i.e. 4·5:1·0. As the concentration of inhibitor rose oxygen uptake was more inhibited than ammonia production, but even with 0·01 M arsenious oxide the ratio was 1·5:1·0, i.e. three times that required for the single step of oxidative deamination.

Isolation of α-ketoglutaric acid. Although the reaction could not be completely blocked after the first stage some α-ketoglutarate probably accumulated in the presence of arsenious oxide, and an attempt was made to isolate it as a 2:4-dinitrophenylhydrazone.

A washed suspension of strain 724 was prepared and the following mixture put up: cell suspension (20 mg./ml.), 10·0 ml.; molar phosphate buffer pH 7·6, 1 ml.; arsenious oxide 0·05 M, 2 ml.; glutamic acid 0·1 M, 6.6 ml.; and water 0·4 ml. This mixture was shaken in the incubator overnight and then the cells were precipitated with trichloroacetic acid and 2 ml. of 1 % (w/v) 2:4-dinitrophenylhydrazine in 2 N hydrochloric acid were added. After standing overnight in the cold a small amount of orange-yellow precipitate formed. Control mixtures from which glutamate or arsenious oxide were omitted gave no yellow precipitate.

The products from three experiments were pooled and purified by the method of Weil-Malherbe & Krebs (1935). The yield was only a few milligrams and it was not possible to recrystallize from ethyl acetate as they had done. Without very careful purification the melting-point was obviously of no help in identifying the compound. Cavallini, Frontali & Toschi (1949) have described a partition chromatographic method of separating the 2:4-dinitrophenylhydrazones of keto-acids and this method was tried on the substance produced by *H. pertussis*. 2:4-Dinitrophenylhydrazones were prepared from known samples of α-ketoglutaric acid, oxaloacetic acid and pyruvic acid and dissolved in 0·02 M phosphate buffer, pH 7·2, at a concentration of 2·5 mg./ml. Using a strip of Whatman's no. 1 filter-paper 12 × 55 cm., and spots containing approximately 20 μg. of each 2:4-dinitrophenylhydrazone satisfactory separation was obtained either in *n*-butanol/water or in *n*-butanol/ammonia. The pyruvate spot moved so much faster than the others that to get adequate separation the solvent front and the pyruvate spot had to run off the end of the paper. Mixtures of the 2:4-dinitrophenylhydrazones of α-ketoglutaric acid and oxaloacetic acid were clearly separated into two spots after approximately 60 hr. at room temperature with either pair of solvents. The unknown hydrazone was put up alongside the hydrazones of α-ketoglutaric acid and oxaloacetic acid. With either solvent the unknown moved as a single spot at the same rate as the hydrazone of α-ketoglutaric acid, and it was, therefore, concluded that the hydrazone isolated was that of α-ketoglutaric acid.

The oxidation of aspartic acid. When suspensions were put up to oxidize aspartic acid very variable rates of respiration were obtained; frequently the rate was little higher than the blank, but occasionally it was 50 %, or more, of the respiration with glutamic acid. Examination of the results of a number of experiments suggested that the higher Q_{O_2} values were given by the denser suspensions and by those put up with least delay after harvesting. To test this, strain 724 was grown as usual, washed, made up into suspensions containing 5 and 15 mg./ml. and put into the manometer flasks as rapidly as possible. The same suspensions were put up again after 6 hr. storage on the bench. While the Q_{O_2} of the stronger suspension declined from 40 to 27·6 that of the weaker suspension fell from 36·0 to 3·5. The activity of the stored weaker suspension was largely restored by the addition of boiled fresh cells and boiled cells added to a weak suspension before storage decreased the deterioration.

The effect of dilution of the cell suspension was investigated by putting up four strengths of suspension to oxidize aspartic acid. Fig. 1 shows that the four suspensions took up 15, 36, 94 and 240 μl. of oxygen in the first 30 min., which implies that the rates (whether the blanks are subtracted or not) increased out of proportion to the strength of the suspension. These observations show a similarity to the behaviour of the serine deaminase of *Bact. coli* (Gale & Stephenson, 1938) and the aspartase II of *Bact. coli* (Gale, 1938). The phenomenon was not studied further, but by using strong fresh suspensions it was possible to perform the oxygen and carbon balance experiments described later.

The relation between the amount of substrate oxidized and the amount of oxygen taken up from other amino-acids. The amino-acids used in these experiments were L-aspartic acid, L-proline, L-serine and DL-alanine, that is, those amino-acids with which oxygen had been taken up most rapidly in preliminary experiments. Cells of strain 724 were grown in Cohen & Wheeler's medium, washed twice in distilled water, and put up in Warburg flasks. The total oxygen uptake and carbon dioxide output was measured in each case. The results of these experiments are given in Table 4, from which it can be seen that even in the absence of an assimilation inhibitor the amounts of oxygen taken up and of carbon dioxide evolved with these amino-acids compared favourably with the theoretical amounts. In another experiment the theoretical amount of oxygen was taken up with DL-aspartic acid. Both isomers of this amino-acid and of alanine must have been oxidized, whereas with serine and proline only half the theoretical uptake occurred with the DL form

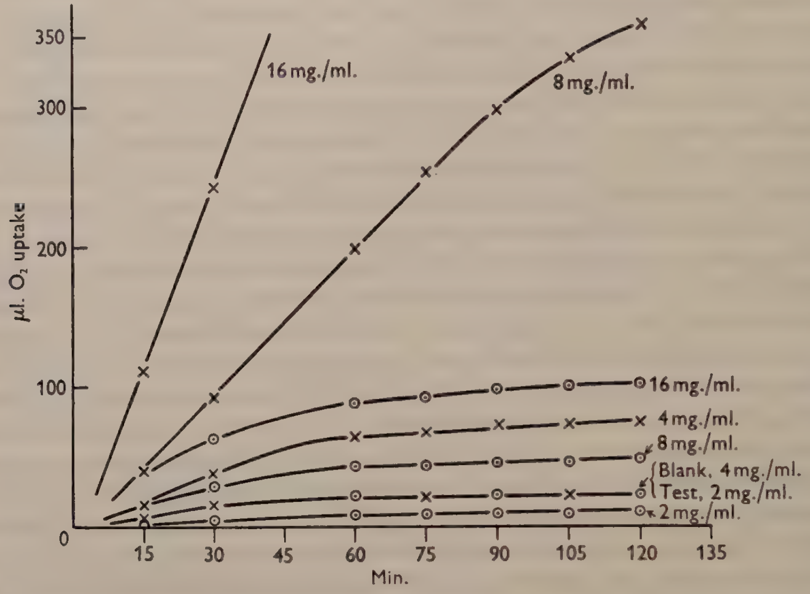

Fig. 1. The oxidation of L-aspartic acid by suspensions of strain 724 of different strengths. ⊙—⊙, blank; ×—×, aspartic acid 0·005 M (final conc.).

Table 4. *Oxidation of various amino-acids to completion by strain 724*

Amino-acid	Sub-strate (μmol.)	O_2 uptake (μmol.)	CO_2 output (μmol.)	NH_3 pro-duction (μmol.)	Substrate:O_2:CO_2:NH_3	
					Observed	Theoretical
L-Aspartic acid	4·0	12·1	16·1	3·32	1·0:3·02:4·02:0·83	1·0:3·0:4·0:1·0
L-Proline	4·0	20·6	19·2	3·88	1·0:5·15:4·80:0·97	1·0:5·5:5·0:1·0
DL-Alanine	4·0	11·1	10·6	4·00	1·0:2·77:2·65:1·00	1·0:3·0:3·0:1·0
L-Serine	4·0	10·2	11·8	4·20	1·0:2·54:2·95:1·05	1·0 :2·5:3·0:1·0

Cells from Cohen & Wheeler's medium made up to 20 mg./ml.

The rate of amino-acid disappearance. By making use of the observed relationships between amounts of oxygen taken up and ammonia formed with each amino-acid it was possible to calculate the rates at which the amino-acids were utilized. To compare the rate calculated from the two measurements a simultaneous determination of oxygen uptake and ammonia production was carried out. In parallel with the Warburg flasks, reaction mixtures were put up in 100 ml. conical flasks, which were shaken in the water-bath, and samples were removed for estimation of ammonia by Nessler's method. The results are given in Table 5 in which the Q amino-acid values are calculated from the ratios given in Tables 3 and 4, assuming 3·2 mol. of oxygen and 0·8 mol. of ammonia per mol. of glutamic acid and the theoretical figures for the other acids. In every case the *Q* value is higher when calculated from the ammonia estimations, and is presumably the more accurate rate for the disappearance of the amino-acid. The rate for aspartic acid is probably low because the necessity for a large crop of cells meant growing the culture for a full 22 hr. whereas the aspartic enzyme is more active in younger cells.

Table 5. *The rates of amino-acid utilization by strain 724 as measured by oxygen uptake and ammonia production*

Substrate	O_2 uptake (μl./hr.)	*Q* amino-acid (μmol./mg./hr.)	NH_3 production (μmol./hr.)	*Q* amino-acid (μmol./mg./hr.)
L-Glutamic acid	245	0·97	6·5	1·85
L-Aspartic acid	50	0·2	1·35	0·385
DL-Proline	130	0·306	1·73	0·49
DL-Serine	70	0·33	1·57	0·448
DL-Alanine	80	0·318	1·55	0·44

Cells from Cohen & Wheeler's medium made up to 4 mg./ml. for glutamic acid and 8 mg./ml. for the others.

The disappearance of glutamic acid from the medium during growth. As glutamic acid is vigorously oxidized by washed suspensions, and as it forms a large part of the amino-acid content of a casein hydrolysate, it was thought advisable to see whether it disappeared from the medium during active growth. To ensure an adequate period of logarithmic growth it was desirable to use a small inoculum, but Cohen & Wheeler's medium, as prepared by us, would not consistently permit the growth of inocula of strain 724 smaller than 10^6 cells/ml. and this inoculum lagged for some 12 hr. This inoculum was accordingly put into 130 ml. medium and incubated at 35° on the shaker; turbidity readings, viable counts, ammonia and glutamic acid estimations were performed at intervals during growth. The results of a typical experiment are given in Fig. 2. Several points emerged from this experiment. Whereas Ungar *et al.* (1950) found that glutamic acid was removed from the medium during 15 day's growth, in this experiment the glutamic acid completely disappeared during the period of logarithmic growth. As might be expected, the viable count increased considerably before any decrease in the glutamic acid content of the medium could be detected by the method used for its estimation. Further, growth was beginning to slow down by the time the glutamic acid had disappeared. The total amount of ammonia produced exceeded that available from the glutamic acid, apart from the considerable amount assimilated into new cell substance.

Growth in amino-acid mixtures

From the preceding results it seemed that *H. pertussis* might grow in a medium containing glutamate as the main source of energy, carbon and nitrogen. Accordingly, media were prepared to Cohen & Wheeler's (1946) formula, but the Casamino acids were replaced by pure amino-acids. Horn, Jones & Blum (1946) described a mixture of amino-acids designed to simulate a casein hydrolysate. As their medium contained 3 μmol. of glutamic acid per ml. and Cohen & Wheeler's medium contained 8–9 μmol. the amounts of the

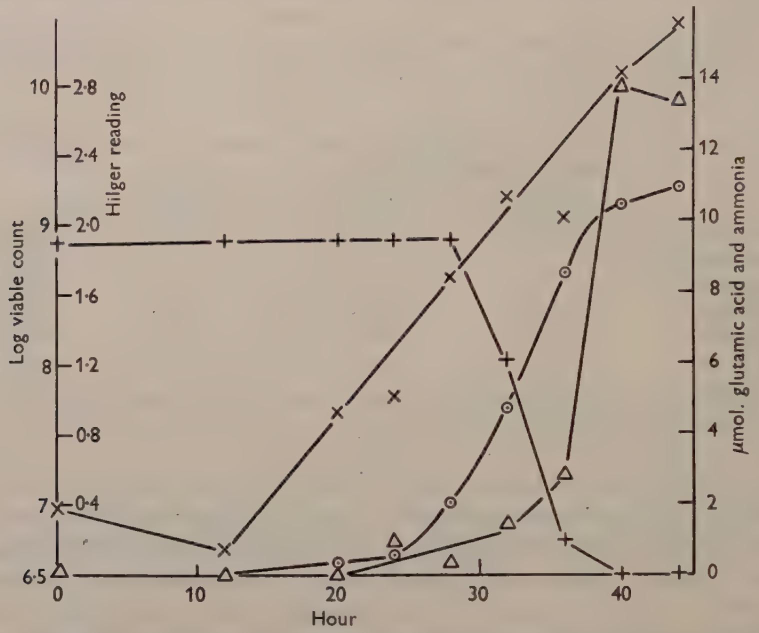

Fig. 2. Growth and utilization of glutamic acid by strain 724. ×—×, log viable count; ⊙—⊙, Hilger reading; △—△, ammonia content of medium; +—+, glutamic acid content of medium.

Table 6. *Growth of strain 724 in amino-acid mixtures*

	A	*B**	*C*	*D*	E
Basal medium (ml.)	100	100	100	100	100
Casamino acids (g.)	.	1·0	.	.	.
L-Glutamic acid (mg.)	.	.	141	.	141
DL-Alanine (mg.)	.	.	.	24	24
L-Aspartic acid (mg.)	.	.	.	36	36
L-Proline (mg.)	.	.	.	42	42
DL-Serine (mg.)	.	.	.	72	72
Growth at 24 hr.	—	++	+±	±	+
Extinction at 48 hr.	0·01	1·54	0·70	0·20	0·64

Basal medium: salts, starch, cysteine and yeast dialysate as in Cohen & Wheeler's medium.

Inoculum: approx. 10^7 washed cells.

* Cohen & Wheeler's medium.

L-isomers were trebled and five media were prepared as shown in Table 6 and distributed in 10 ml. lots in 100 ml. conical flasks. It was hoped that the yeast dialysate would provide essential amino-acids and growth factors. For the inoculum, strain 724 was washed off Bordet & Gengou's medium with quarter-strength Ringer, the cells were spun off and resuspended in quarter-strength Ringer. The flasks were incubated on the shaker at 35°, the growth was recorded at 24 hr. and the densities of the cultures were read in a Hilger 'Biochem' absorptiometer at 48 hr. The results show that glutamate did support good growth and that the other amino-acids, in the proportions used, were not so effective. That growth in the glutamic acid medium was not so good as that in the medium containing Casamino acids was to be expected, but it was surprising that the addition of four other amino-acids to the glutamic acid medium decreased rather than increased the growth. In view of the findings of Dawson *et al.* (1951), it may be that the amount of serine added was inhibitory. This will be investigated later in a study of the nutrition of this organism.

DISCUSSION

This study was undertaken to determine the broad catabolic pattern of *H. pertussis*, and caution is necessary in relating these results obtained in washed suspension to the behaviour of the growing organism. Further, since *H. pertussis* is prone to variation in laboratory subculture, it must be stressed that most of the work was done with a laboratory strain. When tested towards the end of the investigation however, it showed moderate to high virulence. Also some experiments were confirmed with several fresh strains.

With these reservations in mind the following conclusions seem justified· *H. pertussis* is remarkably inactive towards carbohydrates and, as it does not oxidize or ferment starch, the ability of this substance to promote growth under certain conditions must presumably be due to a detoxifying action as suggested by Pollock. On the other hand, *H. pertussis* has a vigorous amino-acid metabolism and four of the acids most rapidly oxidized are those which Ungar *et al.* found to be removed from the medium. They also found that glycine was removed from the medium, but our strain did not oxidize it. The erratic behaviour of suspensions oxidizing aspartic acid suggests that the enzymes or co-enzymes concerned are labile and the aspartic acid metabolism of growing cells may be much more vigorous than our results suggest.

The fact that adding yeast dialysate increased the rate of oxidation of glutamate by cells grown in medium VI suggests that they were deficient in some factor which could be supplied by yeast. Cells from Cohen & Wheeler's medium did not show this phenomenon which seems to indicate a connexion between the rate of glutamate oxidation and the adequacy of the medium. It is hoped to investigate this effect further when the nutrition, and in particular the growth-factor requirements, of *H. pertussis* have been studied.

Glutamic acid stands out as the acid most rapidly metabolized by cell suspensions, both as measured by oxygen uptake and by ammonia production, and as the only one with which oxidative assimilation occurred. Cells which readily oxidized glutamate were much less active towards a number of compounds which might well be intermediates in the oxidation of glutamate. Krampitz & Werkman (1941) showed that oxaloacetate cannot enter intact cells of *Micrococcus lysodeikticus* and if α-ketoglutarate enters *H. pertussis* with difficulty it might be admitted as an intermediate despite its slow oxidation. Slow diffusion into the cells might also explain the failure to oxidize oxaloacetate and the slow oxidation of pyruvate. The isolation of α-ketoglutarate from suspensions metabolizing glutamic acid in the presence of arsenite supports the suggestion that it is an intermediate. With neither glutamic acid nor aspartic acid did we find any accumulation of acetic acid, which is in contrast with Klein's findings with *H. parainfluenzae.*

The rapid disappearance of glutamic acid from the medium during growth and the growth of the organism in a glutamic acid medium strengthen the contention that this amino-acid is probably the principal energy source of the organism, and can possibly serve as sole carbon and nitrogen source, as it does for *Brucella abortus* (Gerhardt, Tucker & Wilson, 1950) and *Clostridium tetanomorphum* (Barker, 1939).

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Isolation of Hydrolytic Products of a Glycerophospho-compound from *Micrococcus pyogenes*

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SUMMARY: The ribonucleic acid fraction of *Micrococcus pyogenes* var. *aureus* (strain Duncan), separated by the method of Schmidt & Thannhauser (1945), contains organic phosphate in excess of that which can be accounted for by a polynucleotide nucleic acid structure. This 'excess phosphate' can be separated from the other phospho-compounds after fractional extraction of *M. pyogenes* in dilute acid or in dilute alkali. Four polyolphosphate compounds occur in the extracts. The main components are α-and β-glycerophosphate, the two other polyolphosphates (giving water-insoluble barium salts; separable chromatographically) occurring in smaller amount. The yields of glycerophosphate show that at least 75 % of the 'excess phosphate' is present in an easily hydrolysed glycerophospho-compound which may also be the origin of the unidentified polyolphosphates. The glycerophospho-compound of *M. pyogenes* accounts for more than one-quarter of the total organic phosphate of the organism. The presence of 'excess phosphate' in a number of Gram-positive organisms suggests that a glycerophospho-compound similar to that of *M. pyogenes* may be of general occurrence in Gram-positive bacteria and in yeasts.

In the course of checking a spectrophotometric method of estimating nucleic acid in intact cell suspensions (Mitchell, 1950*a*) against an adaptation of the method of Schmidt & Thannhauser (1945) developed by Stephenson & Moyle (1949), it was found that although the two methods were in substantial agreement when applied to *Escherichia coli* (strain H), they did not agree when applied to *Micrococcus pyogenes* var. *aureus* (strain Duncan). Comparison of the light absorption at a wave-length of 260 mμ. with the organic phosphate content of the ribonucleic acid (RNA) fraction of the Schmidt & Thannhauser method indicated that in *M. pyogenes* there was an excess of organic phosphate amounting to more than 30 % of that which could be accounted for by a polynucleotide RNA structure (Mitchell, 1950*a*). The occurrence of 'excess phosphate' in the RNA fractions of a number of micro-organisms showed a strong correlation with the capacity for retaining Gram's stain (Mitchell & Moyle, 1950), and it was observed that the amount of 'excess phosphate' in *M. pyogenes* was decreased during growth in the presence of penicillin (Mitchell, 1950*b*; Mitchell & Moyle, 1951).

It was desirable to begin a study of the origin of the 'excess phosphate' of the RNA fraction of Gram-positive bacteria not only to facilitate the elimination of errors in the estimation of RNA, but also to account for the occurrence of a large fraction of the total organic phosphate of these organisms. The present paper describes work on the separation and characterization of fragments of a phospho-compound containing the major part of the 'excess phosphate' of the RNA fraction of *M. pyogenes* var. *aureus* (strain Duncan), cultivated as described by Mitchell (1950*a*).

Definition and measurement of the 'excess phosphate'

In the Schmidt & Thannhauser (1945) method of estimating nucleic acids the cells are treated with cold 5 % trichloroacetic acid to remove the inorganic phosphate and diffusible organic phosphate compounds. The lipid is then extracted with organic solvents and the residue is treated with N alkali at 37° for 15 hr. to depolymerize the RNA. The deoxyribonucleic acid (DNA), which is resistant to the alkali treatment, is reprecipitated with 5 % trichloroacetic acid, leaving the organic phosphate of RNA and inorganic phosphate of phosphoprotein in solution. This solution, referred to as the RNA fraction, contains the 'excess phosphate' in which we are interested.

It was convenient to express the quantity of 'excess phosphate' (XSP) as the number of μmol. organic phosphate in excess of the number of μmol. purine-pyrimidine in the RNA fraction from 1 g. of organism dried *in vacuo* over P_2O_5 at room temperature. The organic phosphate was measured by the difference between determinations of total and inorganic phosphate by micro-adaptations of the methods of Fiske & Subbarow (1925) giving values with a standard deviation of ± 1 % for samples of 3–30μg. P and ± 5 % for samples as small as 0·5μg. P. The measurement of purine-pyrimidine on a comparable micro scale could be carried out only by absorption spectrophotometry. The extinction of the appropriately diluted solutions was determined at 260 mμ. with the Beckman spectrophotometer using quartz cuvettes giving a light path of 1 cm. Owing to lack of information about the proportions of the purine and pyrimidine bases present in the RNA of *M. pyogenes*, there was doubt about the value of the mean molecular extinction coefficient to be used to relate the extinction at 260 mμ. to the mean molecular concentration of purine and pyrimidine base in the nucleotide fragments of the RNA fraction. The published values for the molecular extinction coefficient of the hypothetical nucleotide unit (identical with the mean molecular purine-pyrimidine extinction coefficient) of nucleic acids range from 6500 (Malmgren & Hedén, 1947) to 9000 (Wyatt, 1950). It was therefore desirable to determine the mean molecular extinction coefficient for the purine-pyrimidine as present in the RNA of *M. pyogenes*.

The whole ribonucleoprotein of *M. pyogenes* was isolated by the sonic disintegration and ammonium sulphate precipitation method of Sevag & Smolens (1941), and the organic phosphate content and extinction coefficient at 260 mμ. were measured in neutral aqueous solution. Assuming the molecular concentrations of phosphate and base to be equal, the molecular extinction coefficient was 8200. After depolymerizing the ribonucleo-protein with N-NaOH at 35° for 15 hr., neutralization with HCl and precipitation of the protein with 5 % trichloroacetic acid, the molecular extinction coefficient was found to be 10,000, in agreement with the value of 10,000 found by Wyatt (1950) for a pure yeast RNA preparation after a similar treatment. The mean molecular concentration of purine-pyrimidine in the RNA fraction was therefore estimated using the coefficient 10,000. The standard deviation of the purine-pyrimidine measurements was usually *c.* ± 3 %, the main cause of variation being the occasional occurrence of slight turbidity. We did not consider it advisable to correct for what has been cryptically described by certain workers as 'non-specific absorption'.

As a subsidiary datum, pentose determinations were carried out as by Mejbaum (1939), the period of heating with the orcinol reagent being extended from 15 to 60 min. in order to obtain a more stable colour, the colour being determined photometrically. Pure crystalline ribose was used as a standard, and it was assumed that 65 % of the pentose of the RNA of *M. pyogenes* would be estimated (Schneider, 1945). We confirmed the finding of Valyi-Nagy (1947) that the presence of phosphate causes a depression of the estimated pentose values. In addition to this relatively small effect, however, unknown substance present in *Corynebacterium*, *Neisseria*, certain *Lactobacillus* and *Escherichia* spp., and baker's yeast, changed the colour given by the orcinol reagent and diminished the extinction coefficient by as much as 50 %. It therefore seemed probable that in samples of unknown composition, such as those from the so-called nucleic acid fractions of bacteria, the estimation of pentose would be liable to unaccountable errors. Nevertheless, estimations of pentose were considered to be of use in confirming those of purine-pyrimidine, particularly when significance could be attached to relative changes in the pentose values.

Separation of 'excess phosphate' from the nucleotide components

The 'excess phosphate' might be present in the RNA fraction either as part of the RNA fragments or as a separate compound or group of compounds. Several attempts were made to separate phospho-compounds other than nucleotides from the mono- and poly-nucleotides of the RNA fraction, using ethanol fractionation of the barium and silver salts at various pH values; the separation being followed by the ratio of the extinction at 260 mμ. to the organic phosphate content of the fractions. These attempts, however, gave no indication of the existence of a phospho-compound not containing purine-pyrimidine; nor were we able to obtain paper chromatographic separation of the components of the barium salt fractions.

An alternative procedure to the isolation of components of the whole RNA fraction was the fractional extraction of these components from the cells; for it would be expected that if the 'excess phosphate' were firmly bound to the RNA, the rates of extraction of organic phosphate, purine-pyrimidine and pentose by a given reagent would be the same, whereas if the rates were different the presence of some other phospho-compound might be indicated. The most convenient method for fractional extraction was by hydrolysis in dilute acid or alkaline solution.

Fractional extraction by alkali. In order to minimize the complicating effects of autolytic activity, it was advisable to carry out the extraction at a low temperature and at the highest pH giving a rate of extraction convenient for attempting to observe fractionation. Preliminary experiments showed that 0·1 N-NaOH at 0° gave a suitable rate of extraction of organic phosphate.

In order to stop the hydrolysis when each sample of cell suspension in

0·1 N-NaOH was withdrawn, it was desirable to neutralize with acid. It was found, however, that neutralization with HCl gave erratic results which suggested reprecipitation of the extracted RNA. The effect of bringing the samples to different pH values was therefore examined. A washed suspension of *M. pyogenes* was extracted with 5 % trichloroacetic acid to remove diffusible purine-pyrimidine and phosphate compounds, and washed on the centrifuge with distilled water. The cells were then resuspended at a dry-weight concentration of 10 mg./ml. in 0·1 N-NaOH at 0° for 30 min. Samples of the suspension were neutralized with an equal volume of various acids and centrifuged, and the pH (glass electrode) and organic phosphate content of the centrifugate were measured. The results given in Table 1 showed that at pH values below 6 organic phosphate was removed from the solution. It was observed, however, that this removal was not a simple precipitation of nucleic acid, because, when the cells were centrifuged down from the 0·1 N-NaOH before the pH was lowered, precipitation of organic phosphate did not occur.

Table 1. *Effect of* pH *value on re-absorption of RNA fragments extracted in 30 min. from a suspension of* Micrococcus pyogenes *in* 0·1 N-NaOH at 0°

Solution added to an equal volume of suspension	Final pH value	Amount of organic P left in solution (µmol./g. dry wt. cells)
Water	12·5	86
0·1 M boric acid	9·73	86
0·1 M acetic acid	9·24	84
0·2 M boric acid	8·86	84
0·033 M citric acid	5·90	84
0·05 M citric acid	4·85	59
0·2 M acetic acid	4·70	41
0·1 N HCl containing 10 % (w/v) trichloroacetic acid	3·0	16

Evidently the nucleic acid fragments were adsorbed by components of the cells. This interesting but troublesome effect was eliminated by using an equal volume of 0·1 N acetic acid to neutralize the 0·1 N-NaOH.

The time course of the extraction of purine-pyrimidine, pentose and organic phosphate of the RNA fraction of *M. pyogenes* could now be determined. A washed cell suspension (dry weight at harvesting 4 mg./ml.) was extracted with 5 % trichloroacetic acid at 0° for 30 min. at a dry-weight concentration of 10 mg./ml., centrifuged, and the cell residue was washed once with distilled water. The cell residue was resuspended at the original dry-weight concentration in 0·1 N-NaOH at 0°. Samples, taken at intervals, were neutralized with an equal volume of 0·1 N acetic acid and the cells were centrifuged down from the extracts. Estimations of deoxypentose by the Dische (1930) reaction showed that DNA was not extracted in 0·1 N-NaOH at 0°. The components of the RNA fraction occurring in the extracts were therefore examined directly by determinations of organic phosphate, purine-pyrimidine and pentose. The components of the RNA fraction remaining in the cell residue were similarly examined in the solution obtained by treating the residue with N-NaOH at 35° for 15 hr., neutralizing with N-HCl and reprecipitating the DNA by the addition of 0·2 vol. of 30 % trichloroacetic acid. The progress of the extraction is described in Fig. 1 in terms of μmol. of pentose, purine-pyrimidine and 'excess phosphate' (XSP) per g. of dry organism found in cell residue and extract. Although the pentose values were consistently low for the cell residues, it is evident that the purine-pyrimidine and pentose were extracted about ten times as quickly as the 'excess phosphate', the half-extraction times being *c.* 5 and 50 hr. respectively.

Fractional extraction by acid. Preliminary experiments showed that washed suspensions of *M. pyogenes* were considerably more resistant to acid than to alkaline hydrolysis. Extraction of organic phosphate proceeded slowly in

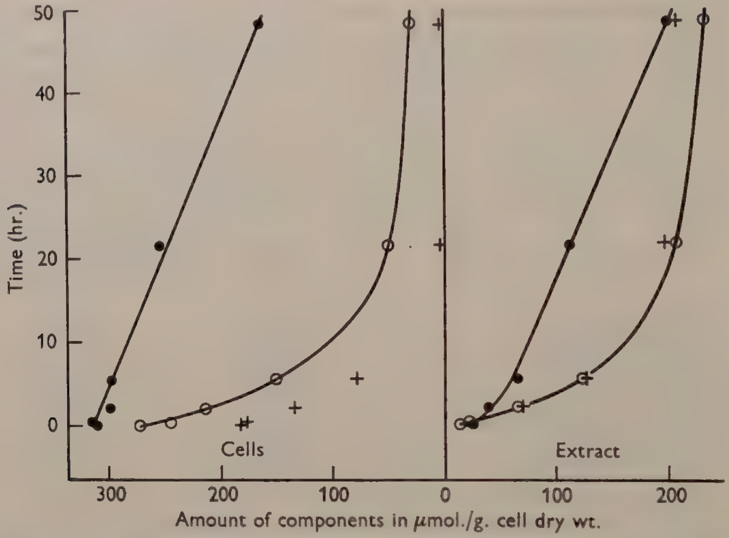

Fig. 1. The time course of the extraction of organic phosphate, purine-pyrimidine and pentose from *M. pyogenes* suspended at a concentration of 10 mg. dry wt./ml. in 0·1 N-NaOH at 0°, observed by determinations on both cells and extract. Expressed as μmol. of purine-pyrimidine or RNA nucleotide units (○——○); μmol. 'excess phosphate' (XSP = total organic P–RNA) (●——●); and μmol. pentose (+ + +) per g. of dry organism.

N-HCl at 0°, and as there was little likelihood of autolytic activity in such an acid solution, the rate of extraction was brought to a convenient level by raising the temperature to 35.° The time course of the extraction of purine-pyrimidine and organic phosphate was determined as follows. Washed *M. pyogenes* (dry weight at harvesting 1 mg./ml.) was suspended in N-HCl at 35° at a dry-weight concentration of 10 mg./ml. Samples were taken at intervals, and the cell residue was centrifuged rapidly from the extract without neutralizing the HCl. The estimations on the cell residues and extracts were carried out as before, pentose estimations being omitted. The initial values for the organic phosphate and purine-pyrimidine content of the extract represented the 'acid soluble' fraction which is usually extracted in 5 % trichloroacetic acid; these values were therefore subtracted from the values subsequently obtained, to give the progress curves. Fig. 2 shows that the half-extraction times were about 10 hr. for the 'excess phosphate' and 2 hr. for the purine-pyrimidine. The total organic phosphate and purine-pyrimidine increased with time because the DNA is extracted by the N-HCl in *c.* 3 hr. This does not, however, affect the relative rates of extraction of phosphate and purine-pyrimidine, for the ratio of phosphate to purine-pyrimidine in the relatively small DNA fraction is near unity. The total 'excess phosphate' of the cells extracted in alkali (Fig. 1) was greater than that of the cells extracted in acid (Fig. 2), because in the latter case the culture of *M. pyogenes* used was harvested at an earlier phase of growth (Mitchell, 1950*b*).

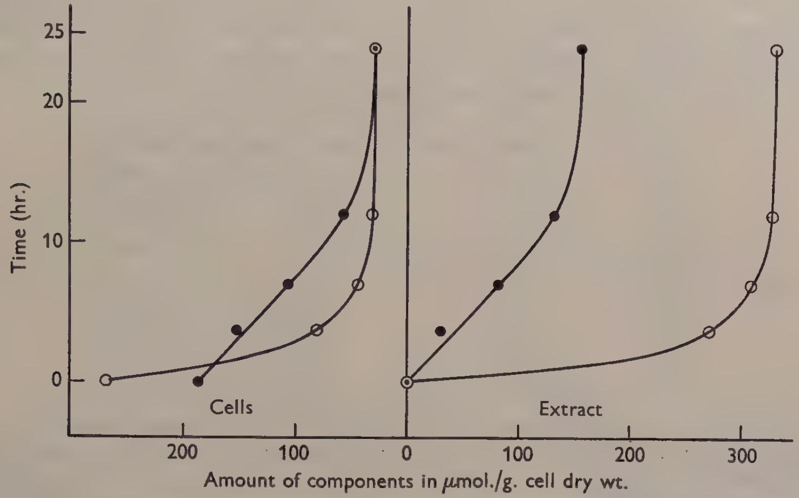

Fig. 2. The time course of the extraction of organic phosphate and purine-pyrimidine from *M. pyogenes* suspended at a concentration of 10 mg. dry wt./ml. in N-HCl at 35°, observed by determinations on both cells and extract. Expressed as μmol. of purine-pyrimidine (○——○) and μmol. of 'excess phosphate' (●——●) per g. of dry organism.

Isolation and characterization of the carriers of the 'excess phosphate'

It was likely that the separation of the carriers of the 'excess phosphate' in *M. pyogenes* would be facilitated by a preliminary extraction of most of the nucleic acid with dilute acid or alkali.

Isolation of barium salt. A crude solution of the 'excess phosphate' carriers was obtained by extracting 10 g. dry *M. pyogenes* with 1 l. 0·1N-NaOH at 0° for 24 hr. to remove RNA, solution of the cell residue in 100 ml. N-NaOH at 35°, neutralizing with 10 ml. 10N-HCl and precipitation of DNA and other contaminants by adding 22 ml. of 30 % trichloroacetic acid followed by 530 ml. ethanol. The solution yielded a water-soluble ethanol-precipitable barium salt containing 6 % P and 5 % N. The nitrogen was present almost exclusively in surface-active, biuret-reacting, non-dialysable substances which could be removed either by foam fractionation or, more easily, by adsorption on activated charcoal. After treatment with 20 g. activated charcoal, the barium salt was repeatedly reprecipitated from water with 2 vol. ethanol. The white barium salt thus obtained, free from sulphur, nitrogen and halogen, and containing 9 % P and 40 % Ba, weighed 230 mg., and represented 670 μmol. of PO_4 out of a total 2500 μmol. of XSP.

General properties of the 'excess phosphate' carriers. The barium salt was soluble in water to the extent of *c.* 5 %. The free acid, prepared by the quantitative precipitation of the barium with sulphuric acid, showed the following preperties. It gave no orcinol reaction for pentose (Mejbaum, 1939) or Dische (1930) reaction for deoxy sugars. A faint Molisch reaction suggested a carbohydrate phosphoric ester, but there was no reducing activity towards either alkaline copper reagents, ferricyanide or dinitrosalicylic acid before or after acid or enzymic hydrolysis. There was no 'rapid furfural', Seliwanoff or Foulger (1932) reaction for sugars or Scherer (1850) reaction for inositol. There was no light absorption between wave-lengths of 2 μ. and 240 mμ. There was a positive acrolein reaction for glycerol.

Table 2. *Titration data for polyolphosphoric acid*

[M]	pH	[Na+]	pK′
3·65	2·48	0·00	1·49
3·62	2·62	0·99	1·45
3·59	2·85	1·98	1·62
3·55	3·40	2·90	—
3·52	5·05	3·85	—
3·48	5·94	4·75	6·17
3·45	6·51	5·65	6·27
3·42	7·16	6·55	6·13
3·38	9·13	7·40	—

[M] = total concentration of polyolphosphoric acid + sodium salt in mg. atoms P per litre.

[Na+] = concentration of sodium ion added to the polyolphosphoric acid as NaOH, in mg. ions per litre.

$$\mathrm{pH} = -\log_{10}[\mathrm{H}^+]$$

$$\mathrm{pK}' = \mathrm{pH} + \log_{10}([\mathrm{M}] + [\mathrm{OH}'] - [\mathrm{Na}^+] - [\mathrm{H}^+]) - \log_{10}([\mathrm{Na}^+] + [\mathrm{H}^+] - [\mathrm{OH}']).$$

The ionization constants were determined by the method of Kumler (1938). A solution of 1·26 mg. of the acid in 1 ml. distilled water was titrated in a polystyrene electrode vessel with CO_2-free 0·1 N-NaOH using 0·01 ml. additions. The pH values were determined with a bulb type glass electrode giving a linear response over the range from pH 1 to 9. The results in Table 2 show two pK′ values, *c.* 1·5 and 6·2, each corresponding to the dissociation of one hydrogen ion per phosphorus atom. In view of the small amount of material titrated, the pK′ values might be in error by as much as ± 0·2 pH unit. According to Kumler & Eiler (1943), glycerophosphoric acid gives pK′ values of 1·4 and 6·4.

Hydrolysis at 100° for 2 hr. in N-HCl or N-NaOH liberated less than 10 % of the phosphate as inorganic phosphate, thus indicating polyhydric alcohol phosphates (Kaplan & Greenberg, 1944). Treatment of a solution at pH 9·5 in 0·02 M bicarbonate buffer containing 0·01 M-$MgCl_2$ with a highly purified preparation of the alkaline phosphomonoesterase of milk (kindly supplied by R. K. Morton) at 35° liberated inorganic phosphate at a rate *c.* 10 % lower than from a control sample of pure β-glycerophosphate, the reaction going to completion.

It was concluded that the unknown phosphate-containing material was a polyhydric alcohol monophosphoric ester (or a mixture of such esters) contaminated by a small amount of carbohydrate. Henceforward this material is referred to as the polyolphosphate fraction.

Chromatographic analysis of the polyolphosphate fraction. Samples of the polyolphosphate fraction, freed from barium by the theoretical amount of sulphuric acid, were run on paper chromatograms following in detail the methods of Hanes & Isherwood (1949), with the results shown in Table 3. The main polyolphosphate corresponded to glycerophosphate, but there was a subsidiary component, representing *c.* 10 % of the total phosphate, running with an R_F value of 0·11 in propanol/ammonia.

Table 3. *Chromatography of the polyolphosphates*

Substance	R_F value
(Solvent: 80 ml. tertiary butanol/20 ml. water + 2 g. picric acid)	
Polyolphosphate fraction	0·69–0·65
α-Glycerophosphate	0·655
β-Glycerophosphate	0·69
Glyceric acid 3-phosphate	0·655
Glyceric acid 2-phosphate	0·68
(Solvent: 60 ml. *n*-propanol/10 ml. water + 30 ml. 20 N ammonia)	
Polyolphosphate fraction	0·305 (strong) 0·110 (weak)
α-Glycerophosphate	0·31
β-Glycerophosphate	0·30
Glyceric acid 3-phosphate	0·21
Glyceric acid 2-phosphate	0·29
Fructofuranose 1:6-diphosphate	0·093
Orthophosphate	0·14

Chromatograms were run at 25° on Whatman no. 1 paper washed with dilute acetic acid. For comparison of the above R_F values with those for other phosphate esters see Hanes & Isherwood (1949).

Chromatographic analysis of the polyols. Dephosphorylation of 25 mg. of the polyolphosphoric acid was carried out with the milk phosphatase under the same conditions as before. The inorganic phosphate was precipitated with excess barium hydroxide, and barium and magnesium removed by passing the solution through a small acid-washed Zeocarb column. The solution was then dried *in vacuo* over P_2O_5 and sodium hydroxide pellets, the latter being required to take up HCl and CO_2. A yield of 12 mg. of a viscous liquid, miscible with water and with ethanol, was obtained. This material was run on paper chromatograms in *n*-butanol/water, using development with ammoniacal silver nitrate according to the method of Hough (1950). Table 4 shows that the main component behaved like glycerol, but there was a subsidiary component, approximately corresponding in amount to the subsidiary polyolphosphate, running with an R_F value of 0·13, the same as xylose. When developed with ammoniacal silver nitrate, the reducing sugars were found to give rise to rapidly appearing brown spots, whereas the polyhydric alcohols developed more slowly, giving black spots. The subsidiary polyol behaved like a polyhydric alcohol during development.

A water-insoluble barium polyolphosphate fraction. During the purification of the polyolphosphate fraction by repeated precipitation of the barium salt from aqueous solution with 2 vol. ethanol, a water-insoluble barium salt fraction was eliminated. This fraction consisted mainly of barium phosphate but some organic phosphate was also present. When examined on propanol/ammonia chromatograms, the organic component of the water-insoluble barium salt fraction was found to correspond to the subsidiary polyolphosphate of the water-soluble barium polyolphosphate fraction ($R_F=0{\cdot}11$). After hydrolysis with the milk phosphatase it gave rise to a polyol chromatographically identical with the subsidiary polyol derived from the water-soluble polyolphosphate fraction ($R_F=0{\cdot}13$ in *n*-butanol/water) and a smaller quantity of a substance running like glycerol. Like the water-soluble barium salt fraction, the water-insoluble barium polyolphosphate fraction showed no reducing activity even towards dinitrosalicylic acid before or after acid or enzymic hydrolysis.

Table 4. *Chromatography of the polyols*

Solvent: *n*-butanol/water

Substance	R_F value
Polyol fraction	0·350 (strong) Bl. 0·130 (weak) Bl.
Propylene glycol	0·64 Bl.
Ethylene glycol	0·57 Bl.
Glycerol	0·35 Bl.
Diglycerol	0·25 Bl.
meso-Erythritol	0·22 Bl.
D-Ribose	0·17 Br.
D-Xylose	0·13 Br.
Mannitol	0·085 Bl.
D-Glucose	0·073 Br.
D-Mannose	0·021 Br.
meso-Inositol	0·007 Bl.

Chromatograms run at 25° on Whatman no. 1 paper washed with dilute acetic acid. Bl. = black spot developing slowly. Br. = brown spot developing rapidly.

Identification of the water-soluble barium polyolphosphate. The water-soluble barium salt fraction was freed from 'water-insoluble' barium polyolphosphate and contaminating carbohydrate by treatment of a 5 % aqueous solution with activated charcoal followed by talc, precipitating the barium salt with 2 vol. ethanol, twice reforming the 5 % aqueous solution (discarding insoluble material) and precipitating the barium salt with 2 vol. ethanol. After drying to constant weight *in vacuo* at 100° over P_2O_5, two different preparations, obtained in a yield of 120 mg. of barium salt from the equivalent to 10 g. of *M. pyogenes* gave on analysis: 9·5 % P; 43·2 % Ba, and 10·1 % P; 44·0 % Ba, corresponding satisfactorily to barium glycerophosphate, $C_3H_7O_6PBa+\frac{1}{2}H_2O$: 9·79 % P; 43·42 % Ba. The identity of the water-soluble barium polyolphosphate as barium glycerophosphate was considered to be proved.

Further chromatographic analysis of the water-insoluble barium polyolphosphates. Samples of the water-insoluble barium polyolphosphate fraction which were judged to be free from glycerophosphate on the basis of chromatograms in propanol/ammonia were found to give as much as 20 % of glycerol after dephosphorylation with the milk phosphatase. This fraction was therefore suspected of being complex. A separation into two components with R_F values of 0·62 and 0·73 was obtained in the *tert.*-butanol/picric acid solvent of Hanes & Isherwood (1949). Using a more acidic solvent consisting of equal volumes of *n*-butanol and N-HCl, a better separation into components with R_F values of 0·22 and 0·41 was achieved. The R_F value of β-glycerophosphate in *n*-butanol/N-HCl was 0·39, and it therefore seemed possible that the spot with $R_F = 0·41$ might represent glycerophosphate formed from the unknown polyol phosphate by the N-HCl. Two-dimensional chromatograms, run first in *n*-butanol/N-HCl and then in propanol/ammonia, showed that both the components appearing on the butanol/HCl chromatograms gave an R_F value of 0·11 when subsequently run in propanol/ammonia. Neither component could therefore have corresponded to glycerophosphate, which has an R_F value of 0·30–0·31 in propanol/ammonia. Further characterization of the water-insoluble polyolphosphate fraction was postponed while larger amounts of material were collected. The work now in progress on this material will be published in due course.

Conclusions. It was evident that *M. pyogenes* must contain a phospho-compound, not extracted by organic solvents, from which glycerophosphate is released on hydrolysis with alkali. The components of the water-insoluble barium polyolphosphate fraction must also have been similarly released either from the same or from some other phospho-compound. In view of the low yields and of the obvious selectivity of our fractionation procedures, it was not known whether the major proportion of the 'excess phosphate' might be accounted for in this way. Moreover, it was uncertain to what extent the treatment with alkali might determine the nature of the phosphate-containing fragments. It was therefore desirable to carry out the fractionation in a more quantitative manner, using as starting material both the crude extract obtained by alkali treatment of *M. pyogenes* and that obtained by an analogous acid extraction.

Quantitative fractionation of alkali- and acid-extracted 'excess phosphate'

Growth and organic phosphate distribution of M. pyogenes. A 4 l. culture of *M. pyogenes* was grown at 25° to a dry-weight concentration of 3 μg./ml. in a 10 l. rotated flask (Mitchell, 1949) in our casein digest medium containing 1 % (w/v) glucose and 0·1 % Marmite. After centrifuging, the cells were washed twice in distilled water, cooled with solid CO_2 in acetone and dried in the frozen state over P_2O_5 in a vacuum desiccator. Estimation of the distribution of the organic phosphate gave the results shown in Table 5, expressed as μmol. phosphate per g. dry-weight organism. For comparison with Table 5, the yields obtained in the extraction and fractionation procedures described below are expressed as μmol. RNA nucleotide units (estimated as purine-pyrimidine) and μmol. XSP (=total organic P–RNA) per g. dry-weight organism. In preparation for the alkali and acid extraction, lipid was exhaustively extracted from two 5 g. samples of dried organism by repeatedly heating under reflux with 95 % aqueous methanol for 1 hr., evaporating to dryness and extracting in a Soxhlet apparatus with sodium-dried ether (Reichert, 1944).

Alkali extraction. A 5 g. sample of dried organism, freed from lipid, was dispersed in 100 ml. 0·1 N-NaOH and held at 0° for 19 hr., neutralized with 100 ml. 0·1 N acetic acid, centrifuged, and the centrifugate discarded. The cell residue was washed once with 0·1 N sodium acetate and dissolved in 50 ml. N-NaOH by incubation at 35° for 15 hr. To this solution, 5 ml. 10 N-HCl and 9 ml. 30 % (w/v) trichloroacetic acid were added, and after 30 min. at room temperature, the precipitate was centrifuged down and discarded. The centrifuged liquid was adjusted to pH 8 with 4 N-NaOH and made up to 150 ml. with distilled water. Yield: RNA = 191, XSP = 246, the units being μmol./g. as defined above.

Table 5. *Distribution of organic phosphate in* Micrococcus pyogenes

Component	Phosphorus distribution: μmol. P/g. cell dry wt.	Phosphorus distribution: % of total P
Free nucleotides and other components extracted in cold 5 % trichloroacetic acid	**119**	**17**
Phospholipid	**38**	**6**
'Phosphoprotein'	**<1**	
DNA	**77**	**11**
RNA (as defined in text)	**197**	**29**
XSP (as defined in text)	**250**	**37**
Total	**681**	**100**

Acid extraction. 5 g. sample of dried organism, freed from lipid, was dispersed in 250 ml. N-HCl and held at 35° for 4 hr., centrifuged, and the centrifugate discarded. The cell residue was washed once with N-HCl and resuspended in 90 ml. N-HCl at 35° for 20 hr. The residue was centrifuged down and discarded, and to the centrifuged liquid 22 ml. 4 N-NaOH and 20 ml. 30 % (w/v) trichloroacetic acid were added. The precipitate was removed by centrifuging and the supernatant liquid adjusted to pH 8 with 4 N-NaOH and made up to 150 ml. with distilled water. Yield: RNA = 25, XSP = 198.

Fractionation. The same fractionation procedure was used for both alkali and acid extracts. To the extract in a 500 ml. flask, 300 ml. ethanol was added. After 2 days, the fine precipitate formed by the ethanol had become attached to the glass and the clear supernatant was quantitatively decanted. To this supernatant, 10 ml. M barium acetate was added; the precipitate of barium salt allowed to aggregate overnight, and was then collected on the centrifuge, washed with 60 % (v/v) aqueous ethanol and dried *in vacuo* over P_2O_5. The barium salt was extracted by grinding 10 times with 5 ml. volumes of distilled water, adjusting to pH 6 with HCl during extraction. Yields thus obtained from the barium salt were: from alkali extract, RNA = 147, XSP = 214; from acid extract, RNA = 25, XSP = 151. The barium salt solution was adjusted to pH 8 in preparation for passage through an acid-washed charcoal column prepared as follows. A suspension of 10 g. of 'B.D.H. animal charcoal' (British Drug Houses Ltd.) in water was packed onto two thicknesses of Whatman no. 1 filter-paper previously covered with a thin layer of talc in a 10 cm. Buchner funnel, and successively washed under gentle suction with 250 ml. 0·1 N-HCl, 100 ml. 5 % KCl, 10 ml. 0·1 M-Ba(OH)$_2$ and 100 ml. 5 % KCl. The solution of the barium salt was drawn slowly through the charcoal column, followed by five lots of 50 ml. of 5 % KCl. The solution was readjusted to pH 8 with barium hydroxide, and the barium salt precipitated by the addition of 4 vol. ethanol and dried *in vacuo* over P_2O_5. Yields: from alkali extract, 145 mg. of barium salt per g. of organism, containing RNA < 1, XSP = 214; from acid extract, 94 mg. of barium salt per g. of organism, containing RNA < 1, XSP = 116.

The barium salt was extracted with 5 ml. of 0·1 N-HCl/100 mg. and the residue was centrifuged down and discarded. The solution was partially separated into water-insoluble and water-soluble barium salt fractions by three successive precipitations of the acidified barium salt with barium hydroxide and precipitation of the soluble barium salt from the supernatant with 4 vol. ethanol. Yields: (as μmol. organic phosphate per g. dry organism) from alkali extract, soluble fraction = 187, insoluble fraction = 17; from acid extract, soluble fraction = 88, insoluble fraction = 31.

In order to obviate the losses inherent in obtaining a complete separation of the soluble and insoluble barium salts, the relative amounts were estimated by determining chromatographically the degree of mutual contamination of the partially separated fractions. The fractions were run on duplicate paper chromatograms in propanol/ammonia, using 0·01 ml. samples of 1 % solutions of the barium salt fractions converted to the free acids by treatment with the theoretical amounts of sulphuric acid. One chromatogram was developed to show the positions of the spots so that the appropriate areas (1 × 1·5 cm.) could be cut out of the duplicate chromatogram, eluted in 5 ml. water, and the organic phosphate determined in the usual way. The weight of phosphorus required in each spot fell in a convenient range between 0·5 and 10 μg. The chromatograms showed that only glycerophosphate and the unidentified polyolphosphates with $R_F = 0{\cdot}11$ were present. The ratio of the weight of phosphate in the glycerophosphate (GP) spot to that in the spot given by the water-insoluble barium polyolphosphates (IP) was as follows: from alkali extract, water-soluble fraction GP/IP > 100, water-insoluble fraction GP/IP = 0·09; from acid extract, water-soluble fraction GP/IP = 9·3, water-insoluble fraction GP/IP < 0·01. Of the 250 μmol. total XSP, the yields of GP and IP were thus: from alkali extract GP = 188, IP = 16, giving a total recovery of 81 %; and from acid extract GP = 80, IP = 39, giving a total recovery of 48 %.

Chromatograms of the water-insoluble barium polyolphosphate fraction in *n*-butanol/N-HCl showed that the two components (R_F values 0·22 and 0·41) were present in this fraction both after alkali and after acid extraction.

Determination of α- and β-glycerophosphate. The glycerophosphate fraction from the acid extract was submitted to further fractionation to remove the remaining water-insoluble barium polyolphosphate. This material and the glycerophosphate fraction from the alkali extract (already free from other polyolphosphates) were assayed for α- and β-glycerophosphate following in detail the periodate oxidation method of Burmaster (1946). The glycerophosphate from the alkali extract assayed as 60 % β- and 40 % α-, while that from the acid extract assayed as 43 % β- and 57 % α-glycerophosphate.

Collection of data. The quantities and yields of the polyolphosphates representing the 'excess phosphate' are set out in Table 6.

Table 6. *The yields of the carriers of the 'excess phosphate'* (*XSP*)

Component	μmol. P/g. cell dry wt.	Recovery of total XSP (%)
From alkali extract		
α-GP	113	45
β-GP	75	30
Total GP	188	75
IP	16	6
Total polyolphosphate	204	81
From acid extract		
α-GP	46	18
β-GP	34	14
Total GP	80	32
IP	39	16
Total polyolphosphate	119	48

GP = glycerophosphate; IP = water-insoluble polyolphosphates.

DISCUSSION

Our experimental observations lead to the conclusion that there is present in *M. pyogenes* an easily hydrolysed glycerophospho-compound of which the phosphate accounts for at least 75 % of the 'excess phosphate' of the RNA fraction and for at least 27 % of the total organic phosphate of the organism harvested towards the end of growth. It is difficult to believe that so large a phosphate fraction should have remained unobserved, but we have been unable to find any previous reference to its existence.

It was not possible to determine the proportions of α- and β-glycerophosphate in the glycerophospho-compound because of the migration of the phosphate between the α and β positions during extraction. The proportions of α- and β-glycerophosphate found after alkali extraction (60 % β and 40 % α) may be compared with the observation of Bailly & Gaumé (1934) that the methyl ester of either α- or β-glycerophosphate gives rise to 2/3 β- and 1/3 α-glycerophosphate on alkaline hydrolysis. In acid, on the other hand, Bailly (1938) found that β-glycerophosphate is converted almost quantitatively to the α-glycerophosphate form. The glycerophosphate isolated from the acid extract (43 % β and 57 % α) might have contained β-glycerophosphate produced during hydrolysis of esters of α-glycerophosphate, or α-glycerophosphate produced from free β-glycerophosphate or both.

The yield of glycerophosphate from the acid extraction was about half that obtained from the alkali extraction. Most of the loss of glycerophosphate from the acid extraction is accounted for by the loss of XSP which occurs during the preliminary acid extraction of RNA. In the alkali extraction, on the other hand, the preliminary extraction of alkali-soluble components resulted in practically no loss of XSP and very little extraction of RNA, the latter being removed on the activated charcoal column at a later stage. This unintentional improvement in the extraction procedure was caused by raising the concentration of the cell suspension in the 0·1 N-NaOH from 10 to 50 mg./ml.

The yield of the two unidentified polyolphosphates was about three times as great from the acid extraction as from the alkali extraction. This difference is susceptible of two possible explanations. It is possible that, as with the relative formation of α- and β-glycerophosphate, the hydrolytic conditions of the extraction procedure determine the amount of the unidentified polyolphosphates formed relative to that of glycerophosphate from a common precursor. On the other hand, the difference between the yields might be due entirely to the selectivity of the extraction procedures. If the latter be the case, the total amount of the 'excess phosphate' which has been accounted for is the sum of the GP obtained from the alkali extraction and the IP obtained from the acid extraction, namely, 227 μmol./g. or 91 % of the total XSP.

The presence of 'excess phosphate' in a number of Gram-positive organisms (Mitchell, 1950*b*; Mitchell & Moyle, 1950) suggests that a glycerophospho-compound similar to that of *M. pyogenes* may be of general occurrence in Gram-positive bacteria and in yeasts, a possibility now under examination in our laboratory.

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The Glycerophospho-protein Complex Envelope of *Micrococcus pyogenes*

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SUMMARY: The glycerophospho-compound which has been shown to account for more than one-quarter of the total organic phosphate of *Micrococcus pyogenes* is present almost exclusively in the particulate fractions of mechanically disintegrated cell suspensions.

The main particulate fraction corresponds to the cell envelope material of Dawson (1949). Three-quarters of the weight of this material is made up of a glycerophospho-protein complex of which the protein moiety resembles silk fibroin in amino-acid composition and in its general properties. The amount of hydroxylamino-acid in the protein of the envelope would be sufficient to bind the polyolphosphoric acids covalently, but the possibility that the polyolphosphoric acids form a polymer which is adsorbed on to a protein matrix is not excluded.

A subsidiary particulate fraction, made up of very small particles containing a high proportion of phospholipid, was found to be otherwise similar in composition to the envelope fraction. It is suggested that in the intact cell, the material of the small particle fraction may form a continuous layer (lipid membrane?) lying beneath the glycerophospho-protein complex envelope (cell wall?).

It has been inferred from observations on the release of glycerophosphate from *M. pyogenes* by hydrolysis in dilute acid or alkali, that a large amount of a readily hydrolysed glycerophospho-compound is present (Mitchell & Moyle, 1951*b*). It was desirable to determine the nature of the complex in which the glycerophosphate is bound, and hence to isolate the intact glycerophospho-compound. The present paper describes the isolation and partial characterization of two particulate fractions of *M. pyogenes* var. *aureus* (strain Duncan) which contain the major part of the glycerophosphate. Some implications of this work have been published (Mitchell & Moyle, 1950).

EXPERIMENTAL

In the course of preparing the ribonucleoprotein of *Micrococcus pyogenes* by disintegrating a cell suspension in distilled water and precipitating the nucleoprotein according to the method of Sevag & Smolens (1941) with 60–66 % saturated ammonium sulphate, it was noted that most of the phosphate in excess of that which could be accounted for as nucleotide units of ribonucleic acid (RNA) (XSP = total organic P–RNA, expressed in μmol/g. cell dry weight) was contained in the water-insoluble debris centrifuged down in 1 hr. at 3000 g, while a smaller portion appeared in the centrifugate fraction precipitated by 60 % saturated ammonium sulphate. Table 1 shows the relevant data and the methods of estimation, the meaning of the symbols being as previously described (Mitchell & Moyle, 1951*b*). It thus appeared likely that the glycerophospho-compound of *M. pyogenes* would be found in the particulate fractions of mechanically disintegrated cell suspensions. Accordingly, we proceeded to prepare and examine the appropriate fractions of *M. pyogenes* grown under the same conditions as before.

Table 1. *The 'excess phosphate' (XSP) content of water insoluble and ammonium sulphate precipitable fractions of mechanically disintegrated* Micrococcus pyogenes

	Phosphate content as μmol./g. cell dry wt.		
Fraction	Total organic P	RNA	XSP
Insoluble	230	—	230
0–60 %*	61·5	46	15·5
60–66 %	62·5	62·5	0
66–80 %	72·0	70·5	1·5

* Figures show percentage saturation with ammonium sulphate required to precipitate the fraction.

Preparation of fractions

Disintegration of cells. The cells were disintegrated by the method of Mickle (1948). The two cylindrical containers (22 mm. internal diameter, 50 mm. internal height, mounted vertically) were each filled with 4 g. of Ballotini glass-balls no. 12, (Chance Bros. Ltd., Birmingham), 10 ml. of distilled water and 250 mg. of freeze-dried organism and vibrated at 50 cyc./sec. for 1 hr. at room temperature. After this treatment, stained smears (Gram) showed less than 1 % intact cells. The froth and underlying opalescent suspension were transferred quantitatively from the containers to a centrifuge tube, together with ten washings of the Ballotini balls with 1 ml. sample of distilled water.

Separation of fractions. The froth was broken by centrifuging for 5 min. at 1000 g and the Ballotini held by the froth were released. The centrifugate was transferred quantitatively to a celluloid centrifuge tube, leaving a small pad of partially disintegrated cells with the Ballotini. The suspension was now centrifuged at 3000 g for 1 hr., and material was deposited in two distinct layers, the lower layer being white and opaque and the upper one yellow and transparent. After decanting most of the opalescent centrifugate, it was possible by gently agitating the tube to redisperse the yellow material in the remaining supernatant liquid without disturbing the underlying layer. The supernatant was removed with a bulb pipette and the surface of the underlying layer was washed with a little distilled water, the latter being added to the residue of partially disintegrated cells. The supernatant and original centrifugate were combined and centrifuged at 15,000 g for 1 hr. to deposit the yellow material from which an almost clear centrifugate could be decanted. Both the white and yellow deposits were submitted to a further similar differential centrifugation after redispersing in small volumes of distilled water. The appropriate parts were combined to give four fractions:

(i) Envelope fraction (see below), corresponding to the opaque white material centrifuging down rapidly at 3000 g.

(ii) Small particle fraction, corresponding to the yellow material centrifuging down at 15,000 g.

(iii) Soluble fraction, corresponding to the centrifugate separated from fraction (ii).

(iv) Residue fraction, consisting of partially broken cells, washings of the surface of the pad of fraction (i) and residues corresponding to fractions (ii) and (i) returned during the second differential centrifuging of fractions (i) and (ii) respectively.

The fractions were dried over P_2O_5 *in vacuo* at room temperature. The weights of the fractions obtained from 1 g. of similarly dried *M. pyogenes* are shown in Table 2.

Table 2. *The weights of the fractions of mechanically disintegrated* Micrococcus pyogenes

Fraction	Weight (mg.)
(i) Envelope	166
(ii) Small particle	84
(iii) Soluble	626
(iv) Residue	102
Total	978

The fractions were obtained from 1000 mg. of dried organism.

Morphology of the fractions

With the assistance of Mr Horne of the Cavendish laboratory, the fractions were examined in the Siemens electron microscope after shadowcasting with gold and palladium, and photographs were taken of representative specimens. Fraction (i) was found to consist of cell envelopes, closely resembling those first observed by Dawson (1949), practically uncontaminated by any other particulate material. Fraction (ii) showed mainly spherical particles 10–50 mμ. diameter. There was a relatively small proportion of flat fragments resembling parts of cell envelopes, but no intact cells or intact cell envelopes. Fraction (iv) contained mostly partially emptied cells, but also some of the material of fractions (i) and (ii).

On examining the fractions with the light microscope, it was found that the cell envelopes could be made visible by staining with amphipathic dyes such as Victoria blue, but that the material of the envelope fraction showed generally a very low affinity either for hydrophilic acidic and basic dyes or for the hydrophobic lipid-soluble ones. While the individual particles of the small particle fraction could not be discerned, the fixed material was found to stain intensely with lipid soluble dyes such as Sudan black.

Distribution of organic phosphate in whole cells and cell fractions

In the procedure adopted for determining the distribution of organic phosphate, most of the manipulations were carried out in a single test-tube to eliminate the losses inherent in transferring the small amounts of material which were available for analysis. Quickfit test-tubes 8 cm. in overall length with B14 sockets were found to be convenient. The material (20 mg. for the whole organism and fractions (i), (ii) and (iv), and 80 mg. for fraction (iii)) was weighed into a stoppered B14 tube. One ml. of 95 % methanol was added, and the material was heated under reflux for 1 hr. to break down the lipid-protein complexes (Reichert, 1944). The methanol was evaporated, and after drying the material *in vacuo* over P_2O_5 for 15 min., it was extracted for 5 hr. with sodium-dried ether in an apparatus designed to obviate the transfer of the material from the Quickfit tube (Mitchell, 1951). The process of heating with methanol and extraction with ether was repeated until no more lipid could be extracted, not more than three extractions usually being required. After evaporating the combined ether extracts to dryness, the lipid was taken up in a small volume of ether, transferred to a weighing tube and dried *in vacuo* over P_2O_5. It was weighed and then redissolved in ether and samples were taken for estimation of phosphate (lipid P). The cell residue in the Quickfit tube was dried and reweighed. It was then dispersed in 2·5 ml. distilled water and 0·5 ml. 30 % (w/v) trichloroacetic acid was added. The residue was centrifuged down and washed twice with 2 ml. distilled water, and the washings were combined with the trichloroacetic acid extract and made up to 10 ml. for estimation of the 'acid-soluble P' on suitable samples. The cell residue was dried and then dissolved by incubation with 2 ml. N-NaOH at 35° for 15 hr. The desoxyribonucleic acid (DNA) was precipitated by adding 0·2 ml. 10N-HCl and 0·44 ml. 30 % (w/v) trichloroacetic acid. After centrifuging down the DNA, the ribonucleic acid (RNA) and 'excess phosphate' (XSP) were determined by estimations of organic phosphate and of the extinction at 260 mμ. of the centrifugate, as previously described (Mitchell & Moyle, 1951*b*). The DNA was measured by the organic phosphate content of the residue after washing once with distilled water and dissolving in 1 ml. N-NaOH. The distribution of the phosphate in the cell fractions is compared with that of the intact cell in Table 3. The envelope and small particle fractions contain a total of 68 % of the XSP of the whole cell and only traces of nucleic acids, while the soluble fraction contains most of the nucleic acids. A further 18 % of the XSP is present in the residue fraction, leaving only 14 % in the soluble fraction. The lipid phosphate of the envelope fraction is very small, but the small particle fraction contains 30 % of the total lipid phosphate. In general, the values obtained for the sum of the fractions agree well with those for the whole cell—indicating that changes due to autolysis during the mechanical disintegration of the cells cannot be serious. The discrepancy is greatest in the case of the RNA values, where the sum of the values for the fractions falls 17 % below that for the intact organism. This drop, which has been repeatedly observed, is due to a decrease in the apparent purine-pyrimidine extinction coefficient, for it is not accompanied by a loss of organic phosphate. For this reason, the XSP values show a corresponding rise in the soluble fraction. Allowing for this error, the XSP value of the soluble fraction would be 35 μmol./g. cell dry weight.

In the evaluation of the RNA content of whole cells from the extinction of the RNA fraction at 260 mμ. it has been our practice not to correct for the relatively insignificant light absorption by substances other than purines and pyrimidines. For the very small RNA values found in the envelope and small particle fractions, however, the absorption of light by substances other than purines and pyrimidines should not be neglected. This was demonstrated by the absorption spectrum of the so-called RNA of the envelope fraction, shown in Fig. 1, which does not correspond to the spectrum of a ribonucleic acid. Hence the already low RNA values for the envelope fraction and probably also for the small particle fraction are erroneously high.

Table 3. *Distribution of phosphate in cell fractions and whole cells of* Micrococcus pyogenes

Phosphate fraction	Fractions				Sum of (i) to (iv)	Whole cell
	(i)	(ii)	(iii)	(iv)		
Acid soluble inorganic	0	2·5	157	1·6	161	166
Acid soluble organic	0	1·9	117	1·2	120	110
Lipid	0·12	11·3	20·5	3·7	35·6	38
RNA	3·1	5·2	137 (170)	19·0	164 (197)	197
XSP	126	43	61 (35)	46	276 (250)	250
DNA	3·0	1·2	59	6·8	70	73

The values given in the body of the Table are the μmol. of phosphate present in the appropriate fraction/g. of dry organism. Values in brackets are corrected for change of apparent extinction coefficient of the RNA fraction at 260 mμ. after disintegration of the cells.

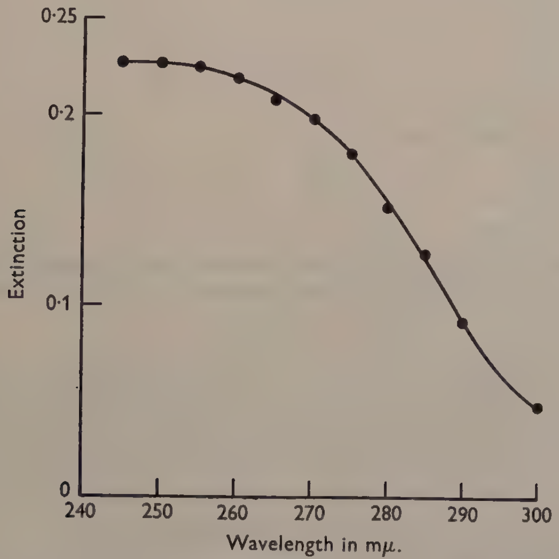

Fig. 1. Absorption spectrum of the so-called RNA of the envelope fraction in acid solution at a concentration corresponding to 1 mg. cell dry weight/ml.

Estimation of polyolphosphates of fractions (i) *and* (ii)

The solution containing the XSP of fractions (i) and (ii) was adjusted to pH 9 after centrifuging down the DNA and taking the small samples required for the estimations described above. Ethanol (2 vol.) was added and the fine precipitate was allowed to adhere to the glass during 2 days. The clear supernatant was decanted and the barium salt of the polyolphosphates was precipitated on adding 10 equivalents of M-$BaCl_2$. The barium salt was centrifuged down, washed once with a little 60 % ethanol and dried. The polyolphosphoric acids were liberated from the barium salt by grinding with 0·1 ml. 0·1N-H_2SO_4/mg., and after removing the barium sulphate by centrifuging, 10 μl. samples of the solution were taken for estimation of total organic phosphate and for chromatographic determination of the ratio of glycerophosphate (GP) to the unidentified polyolphosphates (IP) as previously described (Mitchell & Moyle, 1951*b*). The chromatograms (run in 60 ml. *n*-propanol/30 ml. 20N ammonia +10 ml. water) showed spots corresponding only to GP (R_F=0·30) and IP (R_F=0·12). The yields and proportions of the polyolphosphates are described in Table 4. The recovery of both GP and IP is higher than was obtained from the whole cell, as would be expected. The interpretation of the proportions of the polyolphosphates obtained in the alkali hydrolysate will be dealt with in a later communication.

Table 4. *The recovery of GP and IP from the XSP of fractions* (i) *and* (ii)

Component	μmol./g. dry wt. cells	Percentage recovery of XSP of fraction	Percentage of whole cell XSP
Envelope fraction (i)			
XSP	126	—	51
GP	92	73	36
IP	18	15	8
GP+IP	110	88	44
Small particle fraction (ii)			
XSP	43	—	17
GP	29	68	12
IP	10	23	4
GP+IP	39	91	16

The XSP values are computed by the difference total organic P–RNA. The GP and IP values are determined by estimations of phosphate after isolation of the mixed barium salt and chromatographic separation of the phosphate esters.

Purity of the cell fractions

The fractions of the disintegrated cells have been separated by taking advantage of differences in particle size and density, but the aim has been to obtain a separation of anatomical units of the cell, and it is in this sense that the purity of the fractions must be judged. The electron micrographs showed that the differences in particle size and density were accompanied by distinct morphological characteristics; but only in the case of fraction (i) (the envelope fraction) could the material be recognized as part of an anatomical unit of the cell. The distribution of organic phosphate compounds in the fractions shows that while there are profound differences between the compositions of the envelope and soluble fractions, the small particle fraction differs significantly from the envelope fraction only in containing more phospholipid. We therefore consider it possible that the small particle fraction may have been artificially produced during disintegration of the cells, and that in the intact cell the material of this fraction constitutes part of the cell envelope. The small amounts of nucleic acids found in the envelope and small particle fractions give an indication of the possible degree of contamination of these fractions with the material of the soluble fraction, while the corrected XSP content of the soluble fraction indicates the possible degree of loss of the particulate fractions by solubilization (autolysis?) during the process of cell disintegration.

In order to shed light on the possible modes of attachment of the glycerophosphate, we now proceeded to examine the lipid, carbohydrate and nitrogen-containing components of the particulate fractions.

Composition of the cell fractions

Estimation of nitrogen. Kjeldahl nitrogen was determined by a colorimetric iodomercurate method similar to that of Umbreit, Burris & Stauffer (1949), giving values with a standard deviation of ±3 % for samples of 1–5 μg. Estimations of Van Slyke α-amino nitrogen were kindly made by Dr Haselbach on hydrolysates prepared by heating the material with 5N-HCl at 100° for 24 hr. in sealed tubes.

Estimation of carbohydrate. Total carbohydrate was estimated by the quantitative adaptation of Dreywood's anthrone reaction method developed by Seifter, Dayton, Novic & Muntwyler (1950). For identification of the sugars, the material was hydrolysed by heating at 100° with methanolic-HCl in sealed tubes for 18 hr., evaporation to dryness *in vacuo* over NaOH pellets and heating at 100° for 18 hr. in aqueous N-HCl to hydrolyse the methyl glycosides. The hydrolysates were run on chromatograms in pyridine (1 vol.), water (2 vol.), ethyl acetate (2 vol.) at 25° on Whatman no. 1 paper, the upper layer being used as the moving phase. The chromatograms were developed with 1 % aniline phthalate in butanol, the presence of glucosamine being confirmed in duplicate chromatograms by spraying with 0·2 % ninhydrin in water-saturated butanol. The amount of glucosamine present was estimated very approximately by comparing the intensity of the aniline phthalate and ninhydrin spots with standards of known concentration.

Estimation of lipid. The total loosely bound lipid was estimated gravimetrically after extraction with solvents as described above. Lipid not extracted by this procedure was obtained by carrying out a similar extraction after hydrolysing the material with 5N-HCl at 100° for 24 hr. The lipid thus obtained was freed from traces of glycerol by redissolving in a small volume of ether before being dried over P_2O_5 and weighed.

Results. The estimations of protein, lipid and carbohydrate components of the fractions are shown in Table 5. The protein weight was calculated from the non-lipid Kjeldahl nitrogen weight using a multiplying factor of 6·2, and the weight of polyolphosphoric acid was taken as five times the weight of XSP phosphorus.

It is apparent that the main components of the envelope and small particle fractions are the polyolphosphoric acids, protein and lipid. Part of the lipid is readily separated from the other two components by extraction with organic solvents, but it has not proved to be possible to separate the polyolphosphoric acids from the protein except by methods which hydrolyse the latter. The following solutions were found to exert no solvent action either on the protein or on the polyolphosphoric acids of the envelope fraction in 1 hr.: water at 25° or at 100°; hot or cold methanol, ethanol, *n*-butanol or ether; 5 or 90 % (w/v) aqueous phenol at 25°; 50 % (w/v) aqueous guanidine hydrochloride, pure *N*-dimethylformamide or 70 % (w/v) aqueous *N*-dimethylformamide at 25°; 5 % (w/v) trichloroacetic acid, N acetic acid or 10 N aqueous ammonia at 25°; 0·1 N-NaOH or 0·1 N-HCl at 0°; water/0·1 % (w/v) oxine in *n*-amyl alcohol at 25°. On the other hand, when the envelope fraction was slowly hydrolysed by suspension in N-HCl at 35°, organic phosphate and protein were found to go into solution at a constant rate, about 40 % of the protein and 80 % of the organic phosphate being solubilized in 5 hr. Solubilization of protein and organic phosphate took place at about the same rate on hydrolysis with N-NaOH at 0° or 10 N ammonia at 100° as with N-HCl at 35°.

Table 5. *Composition of envelope and small particle fractions of* Micrococcus pyogenes

Substance estimated	Weight of substance in mg./100 mg. of fraction	
	Envelope fraction	Small particle fraction
Total Kjeldahl N	10·6	9·5
Lipid Kjeldahl N	0·70	3·0
Non-lipid Kjeldahl N	9·9	6·5
Van Slyke α-amino N	10·1	6·9
Protein	62	41
Polyolphosphoric acid	12	8
Lipid (direct extraction)	3·2	22·5
Lipid (from hydrolysate)	4·4	—
Glucose	0·5	1·7
Glucosamine	*c.* 1	*c.* 3
RNA	0·6	2·4
DNA	0·6	0·5
Total recovery	84	79

A more specific hydrolysis of components of the glycerophospho-protein complex might be obtained by enzymic degradation, but so far the material has proved to be resistant. Treatment of the envelope fraction for 3 hr. at 35° with crystalline pepsin at pH 1 or 2, crude trypsin at pH 7·5, crystalline egg-white lysozyme in 0·5 % NaCl at pH 7, intestinal alkaline phosphomonoesterase at pH 9·7 in the presence of 0·001 M-$MgCl_2$, or crystalline pancreatic ribonuclease at pH 7·5 caused no solubilization of phosphate or protein. A preliminary treatment of the envelope fraction in water at 100° for 30 min. made no difference to the action of the above enzymes.

The possibility was considered that the polyolphosphoric acids might be present as a polymer adsorbed on to the protein of the envelope fraction. On the other hand, the evidence available did not exclude the possibility that whether present as a polymer or not the polyolphosphoric acids might be linked covalently to the protein, either directly or through one or more unknown compounds accounting for the 16 % of the weight of the envelope fraction still to be identified.

It was clear at this stage that a knowledge of the approximate amino-acid composition of the envelope fraction would materially assist in our considerations of the possible mode of attachment of the polyolphosphoric acids.

Amino-acid composition of the protein of the envelope fraction

The lipid was extracted from the envelope fraction with organic solvents as before, and 10 mg. samples were heated for 24 hr. with 5N-HCl at 100° in sealed tubes. The hydrolysate was dried *in vacuo* over NaOH pellets and P_2O_5, and redissolved in water to give a concentration of 1 %. The amino-acid composition was determined by running 5, 10 and 20 μl. samples of the hydrolysate on one-dimensional paper chromatograms and measuring the ninhydrin-reacting material eluted from the appropriate areas of the paper by the method of Boissonas (1950). The moving phase used for the chromatograms was phenol saturated with water, and in order to obtain adequate separation of the amino-acids on Whatman no. 1 paper at 25°, the equilibrating phase was varied from equal weights of phenol and water + a trace of KCN to equal parts of phenol and water + 0·1, 0·5 and 3·0 % (v/v) of 20 N ammonia. A high degree of accuracy has been claimed for Boissonas's method, but owing to unaccountable variations which occasionally occurred in our results we considered that significance should not be given to determinations of molecular amounts of amino-acid less than one-twentieth of the amount of glycine present in our hydrolysates. The relative numbers of amino-acid residues are therefore given in Table 6 with glycine written as 20. It is evident that the amino-acid

Table 6. *Approximate amino-acid composition of the acid hydrolysate of the envelope fraction of* Micrococcus pyogenes

Amino-acid	No. of residues	μmol./g. cell dry wt.
Glycine	20	350
Alanine	17	290
Glutamic acid	11	190
Lysine	7	120
Serine	4	70
Threonine	4	70
Leucine + isoleucine	3	50
Aspartic acid	2	35
Valine	1	15
Histidine	<1	—
Arginine	<0·5	—

Other amino-acids present in smaller amount than arginine, not determined.

composition of the whole protein of the envelope resembles that of silk fibroin more closely than that of any other protein for which analyses are available (Tristram, 1949). It differs from silk fibroin mainly in containing relatively much less tyrosine, but more threonine. There is, of course, no evidence that the protein of the envelope fraction can legitimately be regarded as homogeneous, but at all events, a composition resembling that of silk fibroin is the least unexpected of the highly resistant and insoluble material of the protein envelope.

The second column of Table 6 shows the amounts of the amino-acids of the envelope fraction expressed as μmol./g. cell dry weight for comparison with the figures of Table 3. Since the total amount of serine and threonine in the envelope fraction is estimated as 140 μmol. and that of polyolphosphoric acid phosphorus as 126 μmol./g., it would be possible for the polyolphosphoric acids to be linked to the protein through the hydroxylamino-acids. This possibility is now under investigation.

DISCUSSION

Because of the preponderance of protein in the envelope of *M. pyogenes* isolated after mechanical disintegration of the cells, we propose for the present to refer to it as the protein envelope to distinguish it from other layers of less mechanically resistant material which may lie internally or externally to it in the intact cell. A more detailed knowledge of its chemical structure will probably suggest a more specific name for the protein envelope by which it may be distinguished from other envelopes also of a protein nature which may be discovered. It is evident that the polyolphosphoric acids are firmly bound to the protein envelope, either as a polymerized complex (possibly forming a continuous layer) or dispersed in the protein matrix. The phosphate radicals of the polyolphosphoric acids may be covalently linked to the protein, but until this linkage has been proved it is advisable to refer to the compound as a phospho-protein complex rather than as a phospho-protein.

When *M. pyogenes* is treated with 5 % trichloroacetic acid or with phenol or other detergents or organic solvents the osmotic barrier (Mitchell, 1949) is broken and diffusible substances of molecular size not usually exceeding that of the nucleotides escape or can be washed from the cells, although under these conditions the protein envelope is not removed. It seems likely, therefore, that the protein envelope should not be considered as an impervious sheet of peptide chains, but as a pervious network of chains or fibres with an effective maximum pore diameter of *c.* 1 mμ. In the intact cell, this protein envelope presumably acts as the support for the osmotic barrier, part of which may be built into the protein matrix. Cytological evidence indicates the presence of a lipid layer, readily stained by hydrophobic, lipid-soluble dyes such as Sudan black (Burdon, 1946), usually thought to represent the osmotic barrier. This lipid layer lies inside the 'cell wall' which is characterized by a very low affinity for dyes (Knaysi, 1951). In view of the similarity of composition of the non-lipid moiety of the small particle fraction obtained from disintegrated *M. pyogenes*, we suggest that the material of the small particle fraction may be associated with the protein envelope in the intact cell and that the lipid of the small particle fraction may form the layer which is stained by Sudan black. Thus, the protein envelope would correspond to the 'cell wall' and the small particle fraction would contribute the underlying lipid membrane.

Taking into account the weight and composition of the residue fraction, the protein envelope can be estimated to account for *c.* 20 % of the cell dry weight and the small particle fraction for *c.* 10 %. The cells are about 0·7 μ. in diameter at the time of harvesting (Mitchell & Moyle, 1951 *a*) and consequently, assuming the superficial layers to be hydrated to the same extent as the rest of the cell, the envelope fraction would form a surface layer *c.* 25 mμ. thick, while in combination with the small particle fraction it would form a layer *c.* 40 mμ. thick—well below the limit of resolution, but not, of course, below the limit of visibility of the light microscope.

The concept of the protein envelope of *M. pyogenes* as a structural component might be taken as an indication of metabolic inertness, particularly in view of the resistance of the material to enzymic degradation; but it does not follow that because a structure performs a mechanical function that it may not also be involved in a metabolic one, particularly in a system which might be concerned with the transport of metabolites such as amino-acids across an osmotic barrier. It is with this consideration in mind that we are now examining the nature of the glycerophospho-protein complex which, from the envelope fraction alone, accounts for more than 20 % of the total organic phosphate of the cell.

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The Sporulation and Germination of a Strain of *Bacillus megatherium*

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SUMMARY: A strain of *Bacillus megatherium* has been isolated which requires glucose specifically for optimal spore germination. Cyanide, fluoride, iodoacetate, citrate, oxalate, azide, and 2:4-dinitrophenol were ineffective as inhibitors of germination in glucose, while 8-hydroxyquinoline inhibited completely at 10 mm. The inhibitory effect of glucose on sporulation of the organism in liquid and solid media is described.

It has been found (Hills, 1949*a*) that a simple medium containing L-alanine, tyrosine, and adenosine provides practically optimal conditions for germination of certain strains of *Bacillus anthracis*. The germination of a laboratory strain of *B. subtilis* in a buffered medium containing L-alanine only has also been described (Hills, 1950; Powell, 1950). An attempt is now being made to discover the germination requirements of other sporing bacteria; the criterion of germination is again taken as loss of heat resistance, accompanied by change in staining properties and refractivity. In the present report, the sporulation and germination of a strain of *B. megatherium* freshly isolated from air are described. The spores of this organism have been found to require glucose specifically for optimal germination, which proceeds to 80–90 % completion in 30 min. at 35° at spore concentrations up to $2{\cdot}5 \times 10^9$/ml.

A complete systematic investigation of the factors affecting sporulation of this organism has not been made. The effect of adding excess glucose to the medium with and without aeration has been observed, but, as Knaysi (1945*a*) points out, the factors affecting sporulation are complex and interrelated, so that these observations must be regarded as incomplete.

METHODS

Sporulation

It was found difficult at first to obtain suspensions consisting entirely of viable spores. On CCY agar (Gladstone & Fildes, 1940) few spores were formed in 3 days; after incubation for approximately 7 days, CCY cultures produced many spores, but 80 % of these stained densely by the carbol fuchsin-methylene blue technique (Pl. 1, fig. 1) (Powell, 1950) and could be shown, by a comparison of the direct chamber count with the plate count, to be non-viable. The addition of 10 mm. oxalate to the CCY agar medium (Bordet & Renaux, 1930) induced practically complete sporulation in 2 days, but there were still *c.* 20 % of densely staining forms. The first experiments on germination requirements were done using suspensions of this type, after five washings with distilled water.

The meat extract peptone agar medium used by Tarr (1933) for spore production was later found to be much more satisfactory, giving suspensions containing less than 5 % of densely staining non-viable forms (Pl. 1, fig. 2). Growth on this medium was, however, rather poor, although somewhat improved by the addition of small concentrations (5–8 mM.) of glucose (see below). Spores were reaped after 2 days' incubation at 37°, and washed five times with distilled water before use.

Germination

Germination was studied at a spore count of 10^8 to 10^9/ml. and was usually assessed by determining the percentage of spores which became densely staining (carbol fuchsin and methylene blue) during the experiment. This figure for percentage germination agreed with the proportion of spores which had lost their heat resistance as determined by the viable count before and after heating at 60° for 15 min. (Hills, 1949*b*). In some experiments, germination was followed by measurements of turbidity, percentage transmission being determined at 610 mμ. in a Coleman spectrophotometer. The E.E.L. nephelometer (Evans Electro Selenium) was also used and found to be rather more sensitive. In this instrument, a test-tube containing the turbid suspension, illuminated from below, is surrounded by photocells which receive the scattered light. During germination, light scattering and refractivity of the spore are considerably diminished and the instrument readings decrease.

The results which follow suggest that densely staining non-viable spores are produced as a result of growth on media which, although too exhausted or too toxic to permit further growth, still contain an excess of the substances which stimulate germination. It seems that the process involving loss of heat resistance is not directly reversible under these conditions. In this connexion, it has been found desirable with this organism to cool suspensions at 0° as they are reaped and until they have been washed several times. When on one occasion an incompletely washed spore suspension was inadvertently allowed to stand at room temperature for 4–5 hr., it was found to have germinated almost completely.

RESULTS

Germination

It was found that after 30 min. incubation of a 10^9/ml. spore suspension (grown on Tarr medium) in 5 mM. glucose buffered with 33 mM. phosphate pH 7·3, 80–90 % of the cells had become densely staining (Pl. 1, fig. 3). In five experiments, percentage germination was determined in the same sample by both the staining and plating techniques. Agreement between the two methods was satisfactory (Table 1) and it was also found that the germinated cells retained their viability on further incubation for 1 hr. Phosphate, tested in the pH range 6–8, was not stimulatory; germination proceeded equally well in unbuffered glucose, or in glucose buffered with acetate at pH 6 or bicarbonate at pH 8. Glucose solutions were generally autoclaved before use: since Seitz filtered solutions gave exactly similar results, the stimulatory effect of glucose cannot be ascribed to products formed during autoclaving (cf. Emerson, 1948).

Freshly harvested spore suspensions usually germinated satisfactorily in buffered glucose. In a few cases, possibly because of uncontrollable factors in the peptone growth medium, germination did not exceed 50–60 %, even after 14 days' storage of the original suspension at room temperature (cf. Powell, 1950). These suspensions, however, invariably gave at least 90 % germination after they had been heated at 60° for 30 min., and this heat activation was retained indefinitely.

Table 1. *Percentage germination in five samples measured by filming and plating*

Film count		Plate count	
Germination (%)	(Total/germinated)	Germination (%)	(Total/germinated)
88	(306, 269)	90	(882, 792)
59	(512, 303)	69	(759, 525)
70	(521, 367)	68	(738, 504)
79	(573, 450)	76	(601, 459)
77	(592, 453)	74	(601, 447)

Using the spore preparations grown on CCY-oxalate and containing at least 20 % densely staining forms, 50 mM. of either pyruvate, lactate, glycerophosphate or ribose, although they were considerably less effective, produced a result similar to that of glucose. All gave 20–30 % germination in 30 min., and this was not increased by further incubation. When spore suspensions from Tarr medium became available containing less than 5 % of non-viable forms, the stimulatory effect of these compounds was re-tested, and that of other carbohydrates determined. Ribose, glycerophosphate, lactate, and pyruvate were practically ineffective at 70 mM. with these suspensions. Five mM. fructose gave 30 % germination in 30 min., and 50 mM. fructose 75 % in 30 min. Maltose, sucrose, and galactose had no effect at 5 mM., but gave 20 % germination at 50 mM. It seems likely that the effectiveness of fructose at 50 mM. may have been at least partly due to traces of glucose present as an impurity.

The rate of germination in buffered 5 mM. glucose was compared with that in tryptic meat digest, by the turbidity method (Fig. 1). The rate of increase in percentage transmission in glucose was at least as great as, and possibly rather greater than, that in the complex medium. It appears very probable, therefore, that the presence of glucose provides optimum conditions for germination.

The effect of lowered temperature on the rate of germination is shown in Fig. 2. The decrease in light scattering proceeds more slowly at the lower temperature.

Various reagents known to be inhibitors of a stage of respiration or glycolysis have been tested as inhibitors of germination in glucose. In these experiments, the spores were first incubated in buffer for 30 min. with the test substance

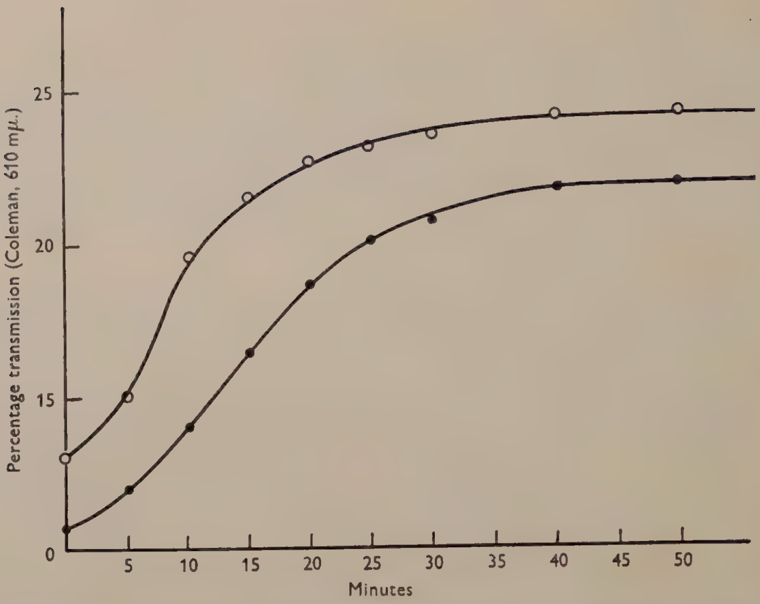

Fig. 1. The germination of *B. megatherium* at 37° in 33 mm. phosphate, 5 mm. glucose (○——○) and in tryptic meat digest (●——●). Measurements of percentage transmission made at 610 mμ.

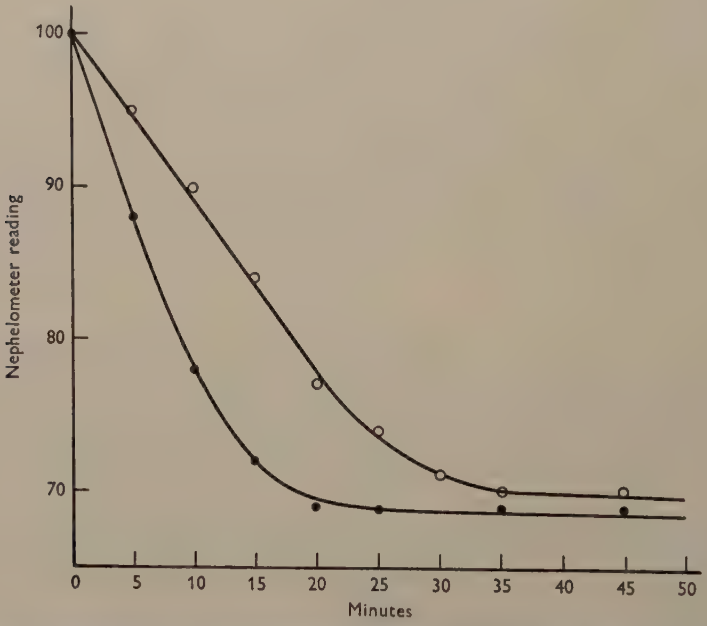

Fig. 2. The germination of *B. megatherium* in 33 mm. phosphate, 5 mm. glucose at 24° (○——○) and 37° (●——●). Measurements of light scattering made in E.E.L. nephelometer.

before glucose was added. One mM. cyanide, 5 mM. azide, 5 mM. 2:4-dinitrophenol, 10 mM. fluoride, 10 mM. iodoacetate, 30 mM. oxalate, and 100 mM. citrate were without effect. On the other hand, as with *B. subtilis* (Powell, 1950), 8-hydroxyquinoline (oxine) was strongly inhibitory, suppressing germination completely at 10 mM., the isomeric 2-hydroxyquinoline being quite ineffective.

To compare the respiration of resting and germinated *B. megatherium* spores, Warburg manometers were used to measure oxygen uptake at 35° in phosphate buffer before and after the addition of glucose to 10 mM. There is a sudden and rapid uptake of oxygen beginning 15-20 min. after the addition of glucose. In a typical experiment, 2×10^9 spores suspended in 40 mM. phosphate buffer pH 7·3 took up less than 10 μl. of oxygen in an hour, but 30 min. after the addition of 10 mM. glucose, a steady oxygen uptake of 50 μl. in 10 min. was recorded.

Sporulation

In the early experiments, described above, it was found that the germination of spores harvested from CCY-oxalate was stimulated, to some extent, by glycerophosphate, pyruvate or lactate. It was expected, therefore, that sporulation might be increased on CCY medium by the omission of glycerophosphate, and decreased on Tarr medium by the addition of glycerophosphate, pyruvate, lactate, or glucose. This was found to be the case. On CCY agar without glycerophosphate, 50 % sporulation occurred in 2 days, although half the spores were densely staining. The addition of sodium lactate (10 mM.) to the Tarr medium decreased sporulation considerably, and gave a high proportion of densely staining forms. There was slight inhibition of sporulation with 100 mM. glycerophosphate. The most marked effect of adding glucose to the Tarr medium was to increase growth. At concentrations of 6 and 12 mM. the addition did not reduce the tendency to sporulation—in fact, sporulation was generally better in these cultures than in the controls. Addition of 25 mM. glucose, however, produced mainly non-sporing cells containing two or more small densely staining bodies. Some spores were produced, but, when free, mostly appeared as densely staining forms. The addition of 50 mM. glucose had a similar, but more pronounced, effect. Pl. 1, fig. 4, shows a typical culture on Tarr medium and Pl. 1, fig. 5, a culture on Tarr medium to which 25 mM. glucose was added; both cultures were examined after 24 hr. incubation at 35°.

As it is generally agreed that the minimum oxygen concentration required for sporulation is higher than that for growth (Knaysi, 1945*a*), the above results might therefore be explained on the basis that heavier growth in the presence of glucose produced 'overcrowding' and consequently oxygen shortage. Another effect of glucose in static culture will be to increase the acidity of the medium, perhaps to a point where sporulation is impossible. It was therefore desirable to compare the behaviour of the organism in shaken (i.e. aerated) culture with and without the addition of glucose. In these experiments, the Tarr medium was used, without agar, and the effect of glucose additions up to 50 mM. was observed. Quadruplicate 50 ml. flasks containing 10 ml. of medium were set up at each concentration, giving duplicate flasks for shaken and static culture. The cultures were examined after 3 days' growth at 35°. In the absence of glucose, sporulation was good in shaken culture, but negligible when the culture was static. Although aeration and addition of glucose improved growth, it was found that glucose was inhibitory to sporulation at concentrations greater than 12 mM. whether the cultures were (*a*) aerated and maintained slightly alkaline at pH 8, or (*b*) static and allowed to become as acid as pH 5. The addition of 6 mM. glucose to shaken cultures did not diminish sporulation, but all the spores formed stained densely. On plating out, less than 1 % of these densely staining spores were found to be viable. The results obtained are summarized in Table 2.

Table 2. *Sporulation of* Bacillus megatherium *after* 3 *days at* 35° *in meat extract peptone medium*

Glucose concentration (mM.)	Static cultures		Shaken cultures	
	pH	Sporulation	pH	Sporulation
0	8·0 8·1	No spores seen	8·1 8·1	Almost complete sporulation, but many (60 %) densely staining forms
6	8·0 7·9	Very granular vegetative cells	8·2 8·2	Complete sporulation and all spores densely staining
12	5·7 5·6	No spores. Growth heavier. Vegetative cells less granular with increasing glucose concentration	8·1 8·1	Poor sporulation. Many granular vegetative cells
25	5·2 5·3		8·0 8·2	No spores. All granular vegetative cells
50	5·2 5·2		8·1 8·1	

Better sporulation was obtained on CCY agar by adding oxalate than by omitting glycerophosphate. It is possible that here another factor is operating: namely, the concentration of available calcium (Bordet & Renaux, 1930). If this is so, sporulation might be expected to increase on the CCY medium on addition of citrate, and to decrease on the Tarr medium on addition of soluble calcium salts. However, it was found that the addition of sodium citrate up to 20 mM. to the CCY medium did not improve sporulation; neither did the addition of calcium chloride up to 13 mM. (0·5 mg. Ca/ml.) to the Tarr medium reduce sporulation. It appears probable, therefore, that the improved sporulation observed on adding sodium oxalate to the CCY medium is due to an effect of the oxalate ion *per se*.

DISCUSSION

Using a microscopic technique, Knaysi (1945*b*) found that germination of *B. mycoides* is stimulated by phosphate buffered glucose. Although no preliminary heat treatment was given, it seems likely that conditions in these experiments were not optimal for the organism tested, since only 30 % germination occurred in 5 hr. Spores of the freshly isolated strain of *B. megatherium* used in the present work require only glucose as an external stimulant for optimal germination. It is possible that the preliminary heat activation required by some spores produces an internal stimulant (Goddard, 1939), the initial concentration of which is regulated by so far uncontrollable factors in the growth medium. Alternatively, it may be suggested that heating facilitates the excretion by the spore of substances which antagonize its germination, the concentration of these substances initially present depending on growth conditions. It has been found recently with resting spores of both this strain of *B. megatherium* and a laboratory strain of *B. subtilis*, that heating at 60° stimulates the excretion of an unidentified substance with a well-defined absorption maximum at 270 mμ. This substance is present in the resting spore, and is excreted in much larger amounts during germination. Its identity and significance are being further investigated.

It is surprising that none of the reagents tested, with the single exception of oxine, inhibited germination in glucose. It has not been proved, however, that these substances enter the resting spore. The failure of the latter to take up dyes suggests that its permeability to other substances may also be relatively low.

The experimental data suggest that, in the presence of certain stimulatory substances, the spore will germinate under conditions which are totally unsuitable for growth, and which, in fact, do not permit the vulnerable germinated form to survive. The impression has been formed in the present study that the germination process is not directly reversible: i.e. if the spore germinates under conditions unfavourable for growth, it cannot revert immediately to its resistant form. It is suggested that the sporulation process requires energy; this is released in some way during germination and must be built up again by a growth phase before the cell, or its daughter cells, can revert to a spore form.

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EXPLANATION OF PLATE 1

Fig. 1. Sporulation after 7 days on CCY agar at 35°.

Fig. 2. Washed suspension from Tarr medium showing one densely staining non-viable spore.

Fig. 3. Spore suspension from Tarr medium after incubation in 5 mM. glucose and 33 mM. phosphate for 20 min. at 35°.

Fig. 4. Sporulation after 24 hr. on Tarr medium at 35°.

Fig. 5. Sporulation after 24 hr. on Tarr medium + 25 mM. glucose at 35°.

Magnification ×1700.

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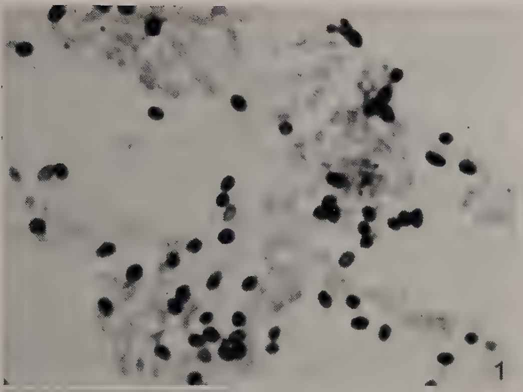
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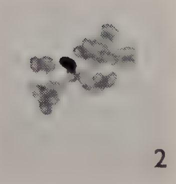
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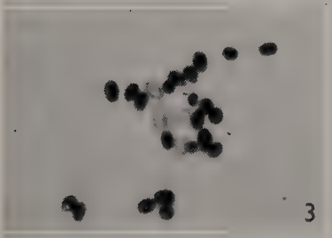
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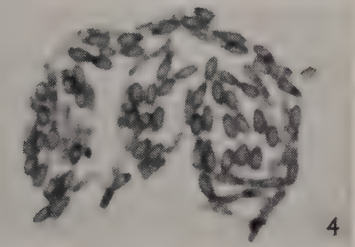
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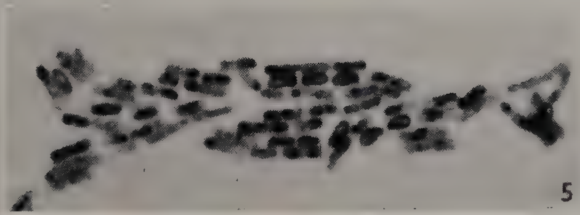
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Figs. 1–5

J. F. POWELL—THE SPORULATION AND GERMINATION OF *B. MEGATHERIUM*. PLATE 1

Brodie, J. & Green, D. M. (1951). *J. gen. Microbiol.* 5, 1001–1009.

A Study of *Shigella flexneri*, *Sh. boydii* and *Sh. sonnei* by Precipitation and Agglutination Reactions

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SUMMARY: Antisera, prepared against 'smooth' variants of the Flexner, Boyd and Sonne types of *Shigella*, were investigated for their agglutinin and precipitin content. The precipitation reactions, employing formamide extracts of the organisms, showed a marked degree of specificity which was lacking in the agglutination reactions except with the Boyd and Sonne types. Cross-precipitation reactions occurred with Boyd's Flexner IV (103) and Flexner Y and also with Boyd's Flexner V (P 119) and Flexner X. Absorption tests, however, revealed each of these four organisms to have a predominant specific precipitinogen.

In previous investigations of the dysentery and colon-typhoid groups (Brodie, 1942, 1948; Brodie & Shepherd, 1949, 1950) it was necessary to check the rough and smooth variants of the organisms employed by colony character, salt-agglutinability, and serological tests. The formamide extract of the smooth variant of *Sh. sonnei* gave a positive precipitation reaction with its homologous antiserum but not with the antiserum to the rough variant; a similar extract of the rough variant reacted with neither. It was decided to investigate the Flexner types of dysentery bacilli for precipitinogens both in the rough and smooth phases.

While this investigation was in process, Gonzales & Morales-Otero (1947) published a paper on the antigenic studies of the *Shigella* by the precipitation reaction. On comparing our available results with theirs we found that ours showed a greater specificity and enlarged the field to include the Boyd types.

METHODS

Preparation of cultures. Smooth variants of the organisms were got by growing cultures on bile-salt citrate rosolic acid agar (Brodie, 1942). Recent batches of sodium tauroglycocholate (British Drug Houses Ltd.) have proved unsatisfactory, and in the preparation of the MacConkey's agar and the bile-salt citrate rosolic acid agar (C.R.A.), 'Bile Salts' (Oxo Ltd.) has been substituted with adequate results.

Rough variants were selected from the smooth strains by repeated subculture on ox-heart extract agar or on MacConkey's agar (1908).

Bacterial suspensions for agglutination tests. The organisms were grown for 24 hr. at 37° on C.R.A., then suspended in 0·25 % (v/v) formalin in 0·85 % (w/v) saline. Further dilution in physiological saline to 2×10^9 organisms/ml. (Brown's scale) was used for all agglutination tests.

Preparation of antisera. The organisms were grown for 24 hr. at 37° on C.R.A., and suspended in physiological saline to give a concentration of 2×10^9/ml.

Graded inocula of the live organisms, commencing with 0·25 ml. and increasing by 0·25 ml. until doses of 2 ml. were reached, were administered intravenously to rabbits at 3- to 4-day intervals. Fresh suspensions were prepared for each inoculation. When test samples showed satisfactory antibody response, the animals were bled out under general anaesthesia, the serum separated, phenol liquefactum added to give a final concentration of 0·5 % (v/v), and the antisera stored at 4°.

Bacterial extracts. Strains were grown for 24 hr. at 37° on C.R.A. from which the rosolic acid had been omitted so that the dye would not tint the end-product. Growth from a 4 in. Petri plate was scraped off and extracted according to the method of Fuller (1938).

Agglutination tests. Twofold dilutions of the antisera were set up. An equal volume of the appropriate suspension was added to each tube and results were read after 4 hr. incubation at 56°. The commencing dilution of each antiserum was 1/25 and the highest dilution showing agglutination with complete clearing of the suspension was regarded as titre. Unabsorbed sera were used throughout.

Precipitation tests. The ring-test technique gave satisfactory readings and was employed in all tests. Readings were taken after 15 min. at room temperature, using the sera used for the agglutination tests.

Absorption of precipitins. Formamide extracts were frozen and dried *in vacuo* over calcium chloride. The dried extract was added in aliquots to the antiserum at 30 min. intervals until the desired absorption was achieved.

Organisms investigated. With the exception of *Sh. sonnei* strains which were isolated in this laboratory, all the organisms studied were obtained from the National Collection of Type Cultures. These are listed in Table 1 along with such information as was supplied with them.

RESULTS

Although the strains employed in this investigation were not identical with those employed by Gonzales & Morales-Otero (1947), it was decided to follow the example of these investigators and arrange the agglutination and precipitation results with the Weil classification (Weil, Black & Farsetta, 1944) as the base-line. This appears to be satisfactory. The remarkable specificity of the results in Table 2 suggests that eleven of the fifteen types have specific precipitinogens, and that, if agglutination occurs to titre with any of the antisera, it is more than probable that a positive precipitation reaction will also be obtained.

The similarities between the Flexner IV (103) and the Flexner Y strain and again between the Flexner V (P119) and the Flexner X, suggested that absorption of precipitins should be attempted. When done, it was found that each of these strains possessed a predominant precipitinogen (Table 3).

Further cultures were examined for their precipitinogen content against these fifteen antisera. They had been mainly classified in the past (Table 1) within the Andrewes & Inman schema (1919), and as none of them gave

Table 1. *Details of cultures investigated*

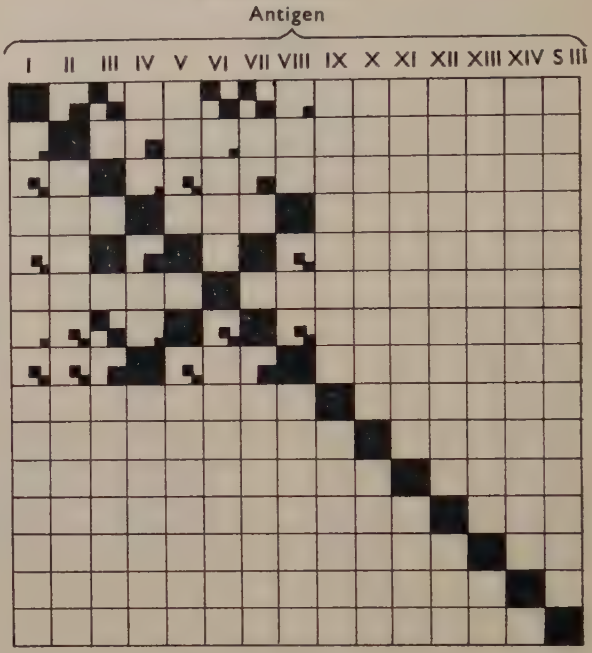
N.C.T.C. no.	Species	Type	Other designation	Date and place of isolation	Isolated or identified by
3	*Sh. flexneri*	1*a*	V, Oxford Flexner	1920 or earlier	.
4832	,,	1*a*	V, Nigeria	1935, Nigeria	J. A. Young, id. W. M. Scott
727	,,	1*a*	V, Lentz Flexner	.	.
4707	,,	1*a*	V, Mott	.	.
5	,,	1*b*	VZ, Stansfield	1923, Southampton	.
2269	,,	?	Munster, Kruse type A	.	VZ by Kalic; Vwx by Scott
4833	,,	2*a*	Willesden	1935, Willesden	W. M. Scott
2272	,,	?	Breslau, Kruse type A	.	VZ by Kalic; Vzx by Scott
2245	,,	var. X	131, Aoki type II	.	Flexner X by Kalic and Scott
2319	,,	3	Kruse type H, Leipzig H511, 218648*a*	.	X by Aoki; Z by Kalic and Scott
2250	,,	3	213, Aoki type X	.	Z by Kalic and Scott
4839	,,	var. Y	Lister 17	.	.
2940	,,	var. Y	F18	Aberdeen	J. Smith
4836	,,	var. Y	Northampton	1934, Northampton	id. W. M. Scott
2270	,,	3	Munster, Kruse type D	.	Y by Kalic
2720	,,	var. X	McCormick	1919 or earlier, Southampton	.
5644	,,	3	Wandsworth	1938, Lister Institute	Miss Steabben, id. Z by Scott
2271	,,	3	Munster-Breslau, Kruse type H	.	Z by Kalic and Scott
2274	,,	3	Breslau, Kruse type H	.	Z by Kalic and Scott
2408	,,	3	Nigeria 27	1927, Nigeria	G. G. Butler, id. Z by Scott
4835	,,	3	Staveley (Leech)	1935, Staveley	Z by Scott
1170	,,	3	Mita type IV	.	Z by Scott
2	,,	var. X	Hughes	1920 or earlier, Southampton	.
4	,,	2*b*	Mountain 2464	1920 or earlier, Southampton	WX by Scott
4834	,,	var. X	Staveley	1935, Staveley	W. M. Scott (Patient sister of 4835)
2246	,,	?	Aoki type III	.	Identical Aoki type V (Kalic)
5451	,,	4*a*	Boyd 103	.	Boyd
5452	,,	5*a*	Boyd P119	.	Boyd
5453	,,	6	Boyd 88	.	Boyd
5454	*Sh. boydii*	1	'170'	.	Boyd
5455	,,	4	'P274'	.	Boyd
5456	,,	2	'P288'	.	Boyd
5457	,,	3	'D1'	.	Boyd
5458	,,	6	'D19'	.	Boyd
5459	,,	5	'P143'	.	Boyd

var. = variant; id. = identified by.

Table 2. *Agglutination and precipitation results with selected strains of* Shigella

(a) Agglutination

Antiserum to N.C.T.C. no.	 Other designation	 Weil type
3	V	I
4833	W	II
5644	Z	III
5451	Flex. 103	IV
5452	Flex. P119	V
5453	Flex. 88	VI
2245	X	VII
4839	Y	VIII
5454	Boyd 1, 170	IX
5456	Boyd 2, P 288	X
5457	Boyd 3, D 1	XI
5458	Boyd 6, D 19	XII
5459	Boyd 5, P 143	XIII
5455	Boyd 4, P 274	XIV
	Sonne	SIII



(*b*) Precipitation

N.C.T.C. no.	Other designation	Weil type	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	S III
3	V	I	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4833	W	II	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
5644	Z	III	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
5451	Flex. 103	IV	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−
5452	Flex. P 119	V	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−
5453	Flex. 88	VI	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
2245	X	VII	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−
4839	Y	VIII	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−
5454	Boyd 1, 170	IX	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
5456	Boyd 2, P 288	X	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
5457	Boyd 3, D 1	XI	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
5458	Boyd 6, D 19	XII	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
5459	Boyd 5, P 143	XIII	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
5455	Boyd 4, P 274	XIV	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
	Sonne	S III	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

■ ▚ ◪ ◪ ◪ ☐ = Agglutination to Titre (T), 2T, 4T, 8T, 16T and <16T

+, − = positive and negative precipitation results.

positive precipitation results with the Boyd types 1–6, agglutination tests were carried out with only the first eight of the fifteen antisera. The agglutination and precipitation results against these eight antisera are tabulated in Table 4.

The strains in Table 4 which gave positive precipitation results both with the Flexner V (P119) and the Flexner X antisera were tested with these sera after absorption of the X precipitin from the former and the P119 precipitin from the latter. The extracts now reacted only with the absorbed Flexner X antiserum, as shown in Table 5.

Table 3. *Results of absorption of precipitin experiments employing freeze-dried formamide extracts*

	Extracts			
Antisera	P119	X	103	Y
(1) P119 unabsorbed	+	+	.	.
(2) P119 absorbed with X extract	+	−	.	.
(3) P119 as (2) further absorbed with P119 extract	−	−	.	.
(4) X unabsorbed	+	+	.	.
(5) X absorbed with P119 extract	−	+	.	.
(6) X as (5) further absorbed with X extract	−	−	.	.
(7) 103 unabsorbed	.	.	+	+
(8) 103 absorbed with Y extract	.	.	+	−
(9) 103 as (8) further absorbed with 103 extract	.	.	−	−
(10) Y unabsorbed	.	.	+	+
(11) Y absorbed with 103 extract	.	.	−	+
(12) Y as (11) further absorbed with Y extract	.	.	−	−
Controls				
(13) P119 absorbed with V (N.C.T.C. 3) extract	+	+	.	.
(14) X absorbed with V (N.C.T.C. 3) extract	+	+	.	.

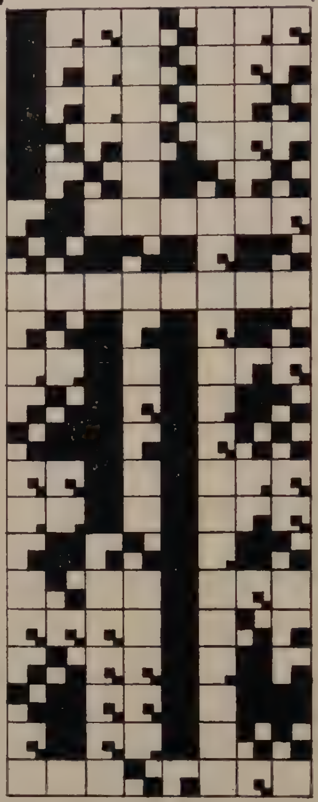
+, − = positive and negative precipitation results

The selection of 'R' and 'S' variants was confined to the Flexner and Sonne strains and the criteria laid down by Arkwright (1920–1) and Braun (1947) were applied. Only two strains—the Sonne type and Flexner W (N.C.T.C. 4833)—fully satisfied the requirements with regard to salt agglutinability, colony characters, and serological specificity. In both instances, extracts of the 'R' variants failed to give precipitation reactions with the antisera to the 'S' variants. None of the other strains, although rough in every sense except that of serological specificity by agglutination tests, completely lost their precipitinogens. Loss of specificity in the precipitation reactions was a frequent finding, cross-reactions developing which were absent with the corresponding 'S' strain. These results are similar to those obtained by Gonzales & Morales-Otero (1947) with his so-called 'old strains'.

DISCUSSION

Seven of the fifteen 'smooth' strains selected as types in Table 2—N.C.T.C. 5453 (Boyd 88), N.C.T.C. 5454 (Boyd 170), N.C.T.C. 5456 (Boyd P288), N.C.T.C. 5457 (Boyd D1), N.C.T.C. 5458 (Boyd D19), N.C.T.C. 5459 (Boyd

Table 4. *Agglutination and precipitation results with other strains of* Sh. flexneri


Agglutination

Antigen: N.C.T.C. no.	Antigen: Other designation	Precipitation: 3 V	4833 W	5644 Z	5451 103	5452 P 119	5453 88	2245 X	4839 Y
4832	V	+	−	−	−	−	−	−	−
727	V	+	−	−	−	−	−	−	−
4707	V	+	−	−	−	−	−	−	−
5	Vz	+	−	−	−	−	−	−	−
2629	Vz, Wvz	+	−	−	−	−	−	−	−
2272	Vz, Vzx	−	+	−	−	−	−	−	−
2319	X, Z	−	−	+	−	−	−	−	−
2250	X, Z	−	−	+	−	−	−	(+)	−
2271	Z	−	−	+	−	−	−	(+)	−
2274	Z	−	−	+	−	+	−	(+)	−
2270	Y	−	−	+	−	+	−	+	(+)
2408	Z	−	−	+	−	(+)	−	−	+
4835	Z	−	−	+	−	+	−	(+)	(+)
1170	Z	−	−	+	−	+	−	(+)	(+)
2	Var. X	−	−	−	−	+	−	−	−
4	WX	−	−	−	−	+	−	−	−
4834	Vwx	−	−	−	−	+	−	(+)	−
2720	Y	−	−	−	−	+	−	+	+
2246	Aoki V,	−	−	−	−	−	−	(+)	+
2940	Var. Y	−	−	−	−	−	−	−	+
4836	Var. Y	−	−	−	−	−	−	−	+

■ ▚ ▟ ▗ ▫ □ = Agglutination to Titre (T), 2T, 4T, 8T, 16T and <16T

+, (+), − = positive, trace positive and negative precipitation results.

Table 5. *Analysis of strains in Table 4 showing cross-reactions with unabsorbed P 119 and X antisera employing the same sera after absorption*

Organisms: N.C.T.C. no.	Organisms: Other designation	Antisera: P 119 unabsorbed	X unabsorbed	P 119 absorbed with X extract	X absorbed with P 119 extract
2274	Z	+	(+)	−	(+)
4835	Z	+	(+)	−	(+)
1170	Z	+	(+)	−	(+)
2270	Y	+	+	−	+
2720	Y	+	+	−	+
4834	Vwx	+	(+)	−	(+)

+, (+), − = positive, trace positive, or negative precipitation results.

P143) and N.C.T.C. 5455 (Boyd P274)—are specific to titre by agglutination and precipitation tests. The agglutination results are in agreement with Boyd (1938, 1940), and with Weil *et al.* (1944), and the precipitation results with Gonzales & Morales-Otero (1947).

The specificity of N.C.T.C. 3 (Flexner V), N.C.T.C. 4833 (Flexner W) and N.C.T.C. 5644 (Flexner Z) by precipitation tests is complete, whereas Gonzales & Morales-Otero (1947) found these organisms to give cross-reactions with Flexners X and Y. Cross-agglutination reactions are marked as one would expect, since unabsorbed antisera only were used. Each organism agglutinates to titre with its homologous antiserum only, except the Flexner Z type which also agglutinates to titre with the Boyd P119 type antiserum. The Flexner Z type, therefore, does not bear out the other results, namely, that if agglutination to titre with any of the antisera occurs, a positive precipitation reaction with the same antisera may be expected. That this is indeed the case, is borne out by the results in Table 3 where other Flexner strains show the same peculiarity as the Z strain in Table 2.

In the past most controversy has centred around the Flexner X and Y types of Andrewes & Inman (1919). These were not considered to be serologically distinct types and were omitted by Boyd (1938, 1940, 1948) and by Wheeler (1944) in their classifications. By agglutination reactions Flexners X and Y are related to Boyd's types P119 and 103 respectively. Gonzales & Morales-Otero (1947), from their precipitation investigations, agree with Boyd and Wheeler in discarding Flexners X and Y, and with the former that they should be replaced by the Boyd types P119 and 103 respectively. Indeed, in Table 2, Boyd type P119 and the Flexner X type show very marked similarities both by agglutination and precipitation tests; the same similarities are evident with Boyd type 103 and Flexner Y. When, however, absorption of precipitins is carried out, it appears that each of these four organisms has a predominant precipitinogen. It seems that Boyd type P119 and Flexner X on the one hand, and Boyd type 103 and Flexner Y on the other, bear a relationship to each other in precipitinogen content similar to the balance of agglutinogens shown by Wilson & Miles (1932) in smooth strains of *Brucella melitensis* and *Br. abortus*. Using specially absorbed agglutinating antisera, Weil *et al.* (1944) found Boyd types P119 and 103 and Flexner types X and Y to be type-specific. Further evidence that this may be so is suggested by the two strains tested, N.C.T.C. 2940 and N.C.T.C. 4836, both 'variant Y' types, which gave positive precipitation results with the Flexner Y type antiserum only (Table 4). That Flexner X possesses a specific precipitinogen has been suggested above and appears to be substantiated by the results with absorbed sera (Table 5). Here certain Flexner strains—Z, Vwx and Y (Kalic)—which show, among other results, cross-reactions with P119 and Flexner X antisera, react only with absorbed Flexner X antiserum and not with absorbed P119 antiserum. The cross-reactions noted in Table 4 are therefore due to the content of these organisms of X precipitinogen and not P119 precipitinogen.

The *Sh. sonnei* type strain employed was one isolated in this laboratory. The smooth variant is type-specific both by agglutination and precipitation tests. Numerous strains isolated here have behaved in an identical manner and none has given cross-precipitation reactions with the antisera available.

The behaviour of the 'R' variants of *Sh. sonnei* and the Flexner W strain (N.C.T.C. 4833) is in accord with Braun (1947), namely, that rough variants lose their precipitinogens. This has also been demonstrated in *Sh. dysenteriae* by Boroff (1949). Variants of the remaining Flexner strains which satisfied the criteria of roughness as defined by Arkwright (1920–1) retained their specific precipitinogen and developed non-specific precipitinogens.

It is essential for consistent results to grow the organisms on special selective media, such as C.R.A. (Brodie, 1942) to ensure a preponderance of smooth variants. Not only is this necessary in order to obtain specific precipitinogens but Takita (1937) showed that phasic variation in cultures of the dysentery bacilli also alters the agglutinogen content of these organisms.

This investigation deals only with the serological peculiarities of certain of the *Shigella*. The nutritional requirements of the same strains are being investigated in this laboratory by our colleague, W. Shepherd, whose findings will be published later. Classical Flexner strains and *Sh. sonnei* are much less exacting in their nutritional requirements than the Boyd types. The latter will not grow to any extent on any synthetic medium so far tested despite the addition of up to seventeen amino-acids and certain growth factors. It appears, therefore, that the Boyd types, as well as exhibiting a well-marked serological specificity, are also more specific in their nutritional requirements than the Flexner and Sonne strains. These findings may tend to add further weight to the proposals of Ewing (1949), who suggests that the *Shigella*, on the basis of serological and biochemical properties, be divided into groups A to E, *Sh. flexneri* comprising group B, *Sh. boydii* group C, and *Sh. sonnei* and *Sh. dispar* group D.

CONCLUSIONS

Among the dysentery bacilli studied and employing smooth variants, fifteen specific precipitinogens are demonstrated.

Boyd type P119 has its own predominant precipitinogen and a moiety of Flexner X precipitinogen; Flexner X possesses the P119 precipitinogen in minor amount and its own specific precipitinogen in major quantity.

Boyd type 103 and Flexner Y bear the same relationship to each other with regard to precipitinogen content as Boyd type P119 to Flexner X.

Precipitinogen and agglutinogen contents do not run in parallel.

'Roughening' produces varying degrees of loss of specificity of precipitinogen content.

Variation is of importance and can be overcome, in part at least, by employing special media favouring the growth of a particular phase.

The authors wish to thank Prof. Tulloch for facilities and criticism, Drs I. C. Whyte and H. Williams for assistance in the early stages of this investigation, Dr W. Shepherd for permission to include data on nutritional requirements, and D. Gellatly for technical assistance. They also wish to thank Brig. J. S. K. Boyd for a subculture of his original D19 strain.

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Staphylococci and their Classification

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SUMMARY: The biochemical and fermentation reactions of 432 strains of aerobic, catalase-positive, Gram-positive cocci were examined in an attempt to produce an orderly classification of the group. The subdivision into *Staphylococcus*, *Micrococcus*, *Gaffkya* and *Sarcina* could not be justified, and, as *Micrococcus* is regarded as an invalid generic name, all aerobic species are placed in the genus *Staphylococcus* with the type species *Staph. aureus* Rosenbach.

The primary subdivision was made on the coagulase reaction, with secondary subdivisions of the coagulase-negative strains based on the production of acid and acetoin from glucose. The characterization of the subdivisions is presented in a quantitative way.

> 'The boundaries of the species, whereby men
> sort them, are made by men.' (Locke, 1689.)

The aerobic catalase-positive Gram-positive cocci present many taxonomic problems which must be resolved before a stable system of nomenclature can be applied to them. We visualize a series of organisms of similar gross morphology that show slight differences in cultural and biochemical reactions, a series that can be likened to a spectrum in that there is a gradual change from the inert non-pathogenic strains to the highly active pathogenic strains.

To test this hypothesis we sought the help of other workers and collected strains from as many sources as possible, and, through the kindness of Dr Ruth E. Gordon, received subcultures of all coagulase-negative strains in the American Type Culture Collection. All strains received, and those in the National Collection of Type Cultures, were put through a standard series of tests which are described in this paper. All cultures were carefully checked for purity, and were dried *in vacuo* (Rhodes, 1950).

METHODS

Optimal temperature for growth. Three nutrient agar slopes, spread with a loopful of broth culture, were incubated at 22, 30 and 37°. Growth was recorded after 24 and 48 hr.

Morphology. Broth cultures incubated at 22° were examined for packet formation. Smears made from agar cultures incubated at the optimal temperature were stained by Lillie's modification of Gram's method.

Biochemical tests were usually made on cultures incubated at the optimal temperature for growth; if an organism grew equally well at two temperatures, the higher temperature was used. Exceptions to this generalization were tests for the liquefaction of gelatin, and certain retests carried out after the main tests had been completed; details are given in the appropriate section.

Pigment. Strains were grown on potato and on nutritent agar at the optimal temperature for 24 hr. and kept at room temperature for a week in diffuse daylight. Pigment was recorded as gold, lemon-yellow, or pink.

Sensitivity to bacteriophages. Two phages, K and W, were spotted on to streak-inoculations on nutrient agar.

Catalase. 'Ten vol.' H_2O_2 was run down the surface of a culture grown on nutrient agar for 24 hr. Gas production, however slight, was recorded as positive.

Methylene blue reductase. One drop 1 % (w/v) aqueous methylene blue was added to 5 ml. broth culture (1 day at 37°, 2 days at 30°, or 3 days at 22°), shaken well to mix, and incubated at 37° for 2 hr. Complete decolorization below the top half-centimetre was recorded as positive, partial decolorization as a trace reaction.

Hydrolysis of urea. A highly buffered medium in which urea was the only nitrogen source (Stuart, van Stratum & Rustigian, 1945), and a feebly buffered nutrient urea medium (Christensen, 1946) were used; the second was controlled by a medium of the same composition save that urea was omitted.

Hydrogen sulphide production. Stroke and stab cultures were made on lead acetate agar. In a recheck lead acetate papers were exposed over a broth culture.

Digestion of serum. Organisms were grown on slopes of Loeffler's medium.

Gelatin liquefaction. Stab inoculations were made in 20 % nutrient gelatin; cultures were incubated for 30 days at 22°.

Coagulase test. Equal volumes of 24 hr. broth cultures were added to 1/10 dilutions of oxalated plasma. Readings were made after incubation at 37° for 4 hr. and after standing overnight at room temperature.

Litmus milk was incubated for 14 days at 37°, 21 days at 30°, or 28 days at 22°.

Fermentation reactions. Peptone water containing Andrade indicator and 1 % (w/v) of 'sugar' was used; final readings were made after 14 days at 37°, 21 days at 30°, or 28 days at 22°. The 'sugars' tested were glucose, xylose, galactose, lactose, sucrose, maltose, raffinose, starch, dextrin, glycerol, mannitol, dulcitol and salicin.

The following tests were made on cultures incubated either for 5 days at 37°, 10 days at 30°, or for 14 days at 22°.

Ammonia production from peptone. Cultures were grown in 0·5 % (w/v) peptone water. An equal volume of Nessler's reagent was added and the colour compared with that produced by the reagent in uninoculated medium of the same batch.

Nitrate reduction. Organisms were grown in nutrient broth containing 0·1 % (w/v) KNO_3. In recheck tests cultures at 37° were incubated for 10 days. Cultures were tested for nitrites by the Griess-Ilosvay method but, as Wallace & Neave (1927) have shown, excess nitrite may obscure a positive result by bleaching the red colour. In the recheck dimethyl-α-naphthylamine was used in place of α-naphthylamine. All negative tests were confirmed by the addition of zinc dust to reduce residual nitrate in the medium (ZoBell, 1932); in a true negative the red colour only appears after the addition of the zinc.

Indole was detected by the Holman & Gonzales method (1923) based on the oxalic acid reaction (Gnezda, 1899).

Methyl red test (M.R.). Cultures were grown in glucose phosphate peptone medium.

Voges-Proskauer tests (V.P.). Two media were used, glucose phosphate peptone medium and a glucose peptone medium without salt or phosphate (Abd-el-Malek & Gibson, 1948). The test was carried out by adding 1 ml. 40 % (w/v) KOH to 4 ml. culture which was then incubated without a plug for 4 hr.; a pink fluorescence was recorded as positive. Barritt's (1936) α-naphthol method was also used on 1 ml. samples of each culture. In the recheck both the foregoing media and an additional medium, glucose peptone medium with NaCl but without phosphate (Smith, Gordon & Clark, 1946), were used; cultures were incubated at 30° for 5 and 10 days and the test was made by Batty-Smith's method (1941).

Utilization of radicals. All tests were made by needle inoculation from a broth culture.

Citrate as sole source of carbon. Koser's citrate medium incubated for 5 days at 37°, 10 days at 30°, or 14 days at 22°. All tubes showing turbidity were confirmed by needle reinoculation into citrate before being recorded as positive.

Ammonium salt as sole source of nitrogen. Agar slopes made up to Hucker's (1948) formula modified by the substitution of litmus for brom-cresol purple.

Sources of strains

After eliminating strains that did not come within the definition of the group being studied, we were left with 462 strains. We had several duplicates or variants, such as the Wood strain and its variants Wood 46 and 86; all these duplicates were removed, and the number of strains to be classified was reduced to 431. Ninety-one strains were known to be of human origin and 43 were from other animals including dogs (15), sheep (14), and cows (5). Milk (20 strains), ice-cream (95), dairy utensils (21), food (20), air (45), water (5), sewage (2) and soil (4) were represented in our series. The source of 85 strains was unknown; many of these were strains from the American Type Culture Collection (ATCC) or from our own Collection (NCTC).

RESULTS

Our aim was to classify aerobic Gram-positive catalase-positive cocci, and we discarded certain strains because they did not have these essential characters. Although some strains were micro-aerophilic, none was rejected because it failed to grow in air. On morphological grounds, however, several strains were rejected because they were either diphtheroids, mainly ellipsoidal but occasionally having one axis more than twice the length of the other, or were strains of *Nocardia*, sometimes coccal and sometimes branched. Among the diphtheroids to be rejected were NCTC 2682 (ATCC 184) *Micrococcus rhodochrous*, NCTC 2683 (ATCC 412) *M. roseus*, NCTC 1390 *Staphylococcus citreus*, NCTC 7650 (ATCC 8411) *M. citreus*, NCTC 7502 *M. cinnabareus*, and NCTC 2559 *M. sphaeroides*. A strain of *M. aurantiacus* (ATCC 387) was coccal when grown at 30°, but produced branching rods at other temperatures. *M. piltonensis* (NCTC 2558) was Gram-negative and was rejected.

We tried to devise a medium that would inhibit the growth of coccal diphtheroids and would allow true cocci to grow. The most successful was a nutrient broth containing 1 % glucose and 2 % sodium taurocholate but occasional diphtheroids grew in it and some cocci were inhibited.

Of the remaining strains it was immediately clear that we were dealing with two entirely different groups; one grew well and produced large opaque colonies, the staphylococcus-micrococcus group; the other grew poorly in air, produced small translucent or semi-opaque colonies, α-haemolytic on horse blood agar, and was very feebly catalase-positive. The second group (α-group) of twenty-nine strains will be described more fully in another paper.

The staphylococcus-micrococcus group was reasonably homogeneous and the cultural characters will be discussed briefly.

Morphology. All strains were Gram-positive in young cultures, but many became Gram-negative as the cultures aged. In size the cells ranged from 0·75 to 2·0μ. and varied on different media. In general the cocci were almost spherical but some showed slight elongation. Pleomorphism was commoner than monomorphism but we were unable to subdivide the group on this basis.

The arrangement of cells was best seen in hanging-drop preparations of broth cultures; paired cocci were the commonest form, and cubical packets the rarest, even in strains named *Sarcina* species. Motility was uncommon (three strains); when present there appeared to be one flagellum per cell. With two strains motility was best seen in young cultures, but one strain was persistently non-motile until the cultures were 5–7 days old. We did not attempt to stain capsules as our earlier attempts had been unsuccessful even with supposedly capsulated strains (Lyons, 1937).

Temperature range and optima. All strains were able to grow at 22°, most grew at 30°, and the majority grew best at 30 or 37°.

Colony form. On nutrient agar colonies were round, low convex, and opaque. Most strains produced colonies with a smooth surface and an entire edge, but a minority had rough surfaced colonies, and a few produced colonies with undulate or crenated edges.

Pigment. Pigment varied and was not always well developed on agar. Some strains refused to grow on potato but others produced highly coloured pigments.

Growth in broth. Nearly all strains produced a uniform general turbidity and a viscous or powdery deposit. When incubation was extended beyond 24 hr. a fine ring pellicle formed on the surface. A few strains failed to produce a general turbidity and these had a granular deposit.

Coagulase tests. All strains were tested with human and rabbit plasma; in addition eighty-four of the coagulase-positive and forty-four coagulase-negative strains were tested with sheep and horse plasma, with the following results:

Plasma	Eighty-four coagulase-positive strains	Forty-four coagulase-negative strains
human	84	0
rabbit	84	0
sheep	61	0
horse	82	0

All plasma was from recently drawn blood, so that age could not account for the smaller number of positives with the sheep plasma.

Sensitivity to phages K and W. Phage K lysed nearly all coagulase-positive and many coagulase-negative strains. W phage was more selective and acted mainly on coagulase-negative strains.

Hydrolysis of urea. Christensen's urea medium (1946) is much more sensitive than that of Stuart *et al.* (1945); it does not give false reactions by alkali production from other constituents in the medium. Most strains were found to hydrolyse urea.

H_2S *production.* Some strains produced a trace of H_2S but few caused extensive blackening of the lead acetate medium.

Digestion of serum. This is not a characteristic of the group as a whole.

Gelatin liquefaction is more rapid in strains of human or animal origin than in those from other sources. With a time limit of 30 days a number of strains are recorded as negative that might have become positive if incubation had been continued (Hucker, 1924*a*).

Litmus milk. Acid production was closely correlated with lactose-fermentation; clots were of the acid and the rennet types.

Fermentation reactions. Most strains produced acid from glucose and several other sugars. In certain 'sugars', such as dulcitol and dextrin, a transient acidity was produced which later reverted to alkalinity.

Ammonia production from peptone. This is an unsatisfactory test; all batches of peptone water give a positive reaction of greater or less intensity when Nessler's reagent is added. We found one batch of Evans peptone that gave only a trace reaction and kept this batch for all subsequent tests. Even in one strain the ability to produce ammonia from peptone seemed to be a variable character and we did not place much reliance on the results we obtained.

Reduction of nitrate. Some irregular results obtained in the first tests were eliminated by using dimethyl-α-naphthylamine. The use of zinc dust was invaluable in checking apparently negative tests.

Indole. None of the strains produced indole.

M.R. Most strains gave a positive reaction but there were several borderline reactions and in these variable results were obtained when the tests were repeated.

V.P. More positives were obtained with the unbuffered media and the α-naphthol test. Batty-Smith's (1941) method is so sensitive that the test may lose its differential value, but it is clearly the most reliable indicator of acetoin production.

Methylene blue reduction. Most strains reduced methylene blue added to broth cultures; we tried the semi-solid methylene blue medium used by Smith *et al.* (1946) but this had no greater differential value.

Utilization of radicals

(*a*) *Citrate as the sole source of carbon.* Few strains were able to grow in Koser's citrate medium.

(*b*) *Ammonium salt as the sole source of nitrogen.* Less than half the strains were able to grow on the medium, and at first it seemed that this test might have differential value. However, repetition of the test did not always give the same result, which suggested that needle inoculation was not always satisfactory in avoiding the carry-over of nutritional factors.

CLASSIFICATION OF THE GROUP

'The genus *Micrococcus* provides what is probably the worst example in bacteriology of uncritical systematic work.' (Abd-el-Malek & Gibson, 1948.)

If we assume that the strains examined are typical of the group they purport to represent, we should be able to use the results as a basis for the classification of the group. If, on the other hand, our strains are not typical or have been derived from too few and insufficiently varied sources, any classification we formulate will not give a true picture of the group as a whole. Some strains had been in culture collections for several years and may not now be typical, but the majority were dried soon after isolation and these are likely to have retained their original characters. As to source, our strains came from man and animals, from food, milk, milk-products, and dairy utensils. To these we added airborne contaminants found on plates of other cultures, strains from air collected by a slit sampler, and a few strains we isolated from water and soil.

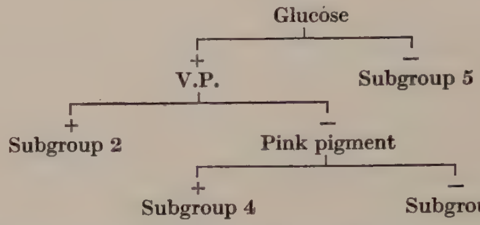
We have mentioned that the α-group appears to be distinct from the staphylococcus-micrococcus group, and we should consider how its removal will affect the variety of our sources. The α-group consists essentially of strains from air samples and strains from dairy utensils, but it should not be inferred that all strains from air and from dairy utensils are of this type; these strains were sent to us because they were unusual. We received strains from dairy utensils and dairy products from Dr T. Gibson's collection, and these fall naturally into our staphylococcus-micrococcus group, as do those from Dr G. J. Hucker and from the American Type Culture Collection. We feel justified, therefore, in assuming that our strains are not only typical but are representative of the group as a whole.

Our next task is to decide how the staphylococcus-micrococcus group should be subdivided. One line of cleavage is clear, that made by the coagulase test, which separates the toxigenic and pathogenic strains sharing a series of common antigens (Hobbs, 1948) and sensitive to a series of specific bacteriophages (Wilson & Atkinson, 1945) from the non-toxigenic and non-pathogenic of diverse antigenic make-up (Cowan, 1938) and not susceptible to bacteriophages specific for coagulase-positive strains. We choose the coagulase test because it is simpler to demonstrate than are the specific toxins.

We attempted to subdivide the coagulase-positive subgroup; Cowan (1938) suggested that the V.P. reaction might be useful, but by using different media and more sensitive reagents we found that nearly all animal strains produce acetoin. Coagulase-positive strains can be subdivided by the toxins they produce; human strains seldom produce β-toxin and many animal strains do so, but we do not think that this subdivision is profitable, and we are content to leave the coagulase-positive strains as a fairly homogeneous subgroup entitled to specific rank. We do not consider pigmentation to have even varietal value in this species.

Division of the coagulase-negative subgroup presented greater difficulty; the ability to utilize NH_3 salts was found useful by Hucker (1924*b*, 1948), while Abd-el-Malik & Gibson (1948) divided their strains primarily on heat resistance and fermentative ability. We did not use a heat-resistance test because we were not convinced that the methods in use were sufficiently reliable to give reproducible results, but the ability or inability to ferment sugars seemed to us to be a good starting-point in the subdivision.

We drew up a preliminary classification using NH_3 salt utilization and the M.R. test, but the two distinguishing characters were variable in too many strains; we then rearranged our fermenting strains into a series of subgroups divided primarily on acetoin formation, with lactose and mannitol fermentation as secondary differential characters. Again, the groupings were unsatisfactory, and, apart from the differential criteria themselves, there was little homogeneity within a group. Finally, we divided our coagulase-negative strains according to the following scheme, in which the differential criteria are characters that seldom show variation when strains are re-examined; this grouping brings together strains with several characters in common. Like all other classifications of the cocci, it is arbitrary and artificial.



Characteristics of the subgroups so far defined are shown in Table 1; subgroup 1 consists of coagulase-positive strains; subgroups 2–5 of coagulase-negative. The α-group is included for comparison with the other subgroups. The figures show the percentage of strains producing the reaction indicated; caution should be exercised in accepting these too literally as most are based on small numbers within each subgroup.

Correlation of grouping and source of strains

We did not attempt to correlate our groups with the source of the strains until the grouping seemed satisfactory on other grounds. We believe that the source of a culture should not have a place in any bacterial classification scheme, but present Table 2 for those whose opinion differs from ours. Subgroups 1 and 2 were chiefly represented by strains from animals or from material likely to be contaminated by animals, subgroup 3 from dairy products. Subgroups 4 and 5 were nearly all strains of unknown origin from culture collections, and many of these were named species.

Distribution of named strains

Apart from the strains freshly isolated from ice-cream, dairy utensils, and air, most of the strains had been assigned to particular species. The cultural characters of the named strains did not always correspond to the description of the species. As many of these strains are available in culture collections, the more important are listed in Table 3. Most strains in subgroup 1 were named *Staph. pyogenes* or *Staph. aureus*, and are not shown in the table. We did not have any typical strains of *Micrococcus tetragenus*.

DISCUSSION

The value of a classification lies in its usefulness; can it be said that any classification of these aerobic cocci will serve a useful purpose? The medical bacteriologist wants only to classify pathogenic strains, the dairy bacteriologist those of economic importance in milk, dairy products, and dairy hygiene. Should each type of bacteriologist be allowed to develop his own classification or should some attempt be made to classify within one system the group as a whole? We believe that this attempt is worthwhile, and we have made it.

Table 1. *Cultural characters of subgroups*

	Subgroup					
	1	2	3	4	5	α
No. of strains	143	149	63	16	31	29
Opt. temperature: 22°	0	0	0	25	10	0
30°	2	7	38	69	42	34
37°	98	93	62	6	48	66
Pigment: none	25	55	51	0	29	100
gold	69	38	30	0	13	0
yellow	6	7	19	0	45	0
pink	0	0	0	100	13	0
NH_3-salt utilization	0	27	51	88	74	52
M.R., positive	99	88	68	0	0	93
V.P., positive	94	100	0	0	0	0
NH_3 from peptone	98	88	92	56	84	0
H_2S produced	27	14	10	0	29	0
Nitrates reduced	97	73	83	75	26	0
Methylene blue reduced	100	99	94	19	71	83
Litmus milk: acid	94	62	73	0	0	93
acid clot	56	35	41	0	0	0
digestion	4	5	2	0	0	0
litmus decolorized	40	22	27	0	0	0
Gelatin liquefied	100	51	63	6	71	0
Serum digested	26	6	0	0	0	0
Coagulase-positive	100	0	0	0	0	0
Acid from: glucose	100	100	100	100	0	100
xylose	3	11	62	0	0	41
galactose	98	83	78	0	0	97
lactose	98	67	81	0	0	97
sucrose	100	99	83	44	0	97
maltose	100	98	87	13	0	100
raffinose	3	1	17	0	0	31
starch	6	4	3	19	0	27
dextrin	77	32	22	19	0	38
glycerol	94	95	79	6	0	97
mannitol	97	75	70	25	0	69
dulcitol	3	2	3	0	0	0
salicin	22	13	51	6	0	83
Urea hydrolysed	85	77	67	44	26	0
Sensitivity to phage K	83	22	21	0	0	0
Sensitivity to phage W	1	9	0	0	0	0

Figures show percentage of strains giving the reaction.

Table 2. *Source of strains*

	Subgroup					
Source	1	2	3	4	5	α
Human	60	25	2	0	4	0
Other animals	34	4	5	0	0	0
Ice-cream	4	66	25	0	0	0
Milk and dairy utensils	7	5	13	0	0	16
Food	4	11	4	0	1	0
Air	1	26	5	0	1	12
Water, soil, sewage	0	2	4	0	4	1
Unknown	33	10	5	16	21	0

Table 3. *Distribution of named strains in the subgroups*

	Collection nos.		
Name	ATCC	NCTC	Subgroup
M. agilis	998	2676	5
M. agilis	—	3874	5
M. candicans	8406	7558*	2
M. candicans	8407	7559*	2
M. candicans	8456	7733	5
M. candicans	—	1657	5
M. caseolyticus	8460	7735*	1
M. cereus	—	3881	5
M. conglomeratus	401	2677	3
M. corallinus	966	7509*	4
M. cremoris-viscosi	—	963*	3
M. flavo-roseus	397	7528	4
M. flavus	400	2678*	5
M. flavus	10240	7743*	5
M. freudenreichii	407	2679*	5
M. freudenreichii	—	3880*	5
M. lactis-viscosus	—	3873*	2
M. liquefaciens	—	2654	2
M. luteus	379	2680*	5
M. luteus	398	2681*	5
M. lysodeikticus	4698	2665	5
M. roseus	144	7511	4
M. roseus	177	7512	4
M. roseus	178	7513	4
M. roseus	179	7514	4
M. roseus	185	7515	4
M. roseus	388	7516	4
M. roseus	412	7517	4
M. roseus	416	7518	4
M. roseus	417	7519	4
M. roseus	418	7520	4
M. roseus	516	7521	4
M. roseus	534	7522	4
M. roseus	9815	7738	4
M. rubens	186	7523	4
M. sulphureus	—	1631	3
M. tetragenus	6007	7459*	5
M. tetragenus	—	951	2
M. tetragenus	—	7501	2
M. ureae	408	2684*	5
M. varians	399	2685	3
M. varians	—	7281	3
M. vernae	10209	7742	2
Sar. aurantiaca	—	952*	3
Sar. flava	—	4351	3
Sar. flava	—	7495	3
Sar. lutea	—	196	5
Sar. lutea	—	611	5
Sar. ureae	—	4819	5
Staph. citreus	—	2801*	5
Staph. citreus	4012	5954	1
Staph. citreus	9510	7739*	5
Staph. epidermidis	155	5955	2
Staph. epidermidis-albus	—	1400	2
Staph. epidermidis-albus	—	1401*	1
Staph. flavo-cyaneus	8676	7011	5
Staph. salivarius	—	189*	2
Staph. viscidus	—	5538*	2

* These strains differ significantly from the usual description of the species named.

We must first consider the group as a whole and decide on its rank; is it sufficiently homogeneous for generic status? We would remove the α-group of cocci for these seem to differ fundamentally from the other strains; a reasonably homogeneous group remains but should it be subdivided at the generic level? Cowan & Shaw (1949), in describing their classification of *Staphylococcus*, discussed this problem, particularly in relation to restricting the genus *Staphylococcus* to the potentially pathogenic strains. We searched for some other characteristic to correlate with coagulase-production, toxigenicity, and pathogenicity, and we have failed to find it; we conclude, therefore, that coagulase-positive and coagulase-negative staphylococci should for the present be included in the same genus. Is there any justification for subdividing the coagulase-negative strains at the generic level? We do not think so, and suggest that the whole group should be considered as one genus.

Cummins & Cummins (1913) found fermentation tests were unreliable and inconstant; Ferguson Wood (1950) has shown what every worker has suspected, that when a pure culture is plated it is unusual for two colonies to give exactly the same reactions when individual colonies are put through a number of fermentation and biochemical tests. It is clear, therefore, that no hard-and-fast lines of demarcation can be drawn between different subgroups and that each must be defined on a quantitative basis.

The subdivisions of our classification were made after we had found that the species recognized by Hucker (1924*b*, 1928, 1948) were meaningless. Abd-el-Malik & Gibson (1948) suggested that acetoin production was a valuable differentiating criterion; we found this to be one of the most constant characters of a strain, and, after separating the non-fermenting strains, used it as the main differential test.

It is impossible to make a direct comparison between our classification and that of Hucker as we used different criteria, but, by an exchange of cultures with Dr Gibson, fifteen strains were classified according to our two schemes. Six strains labelled groups II A, II B or II C by Gibson fell into our subgroup 5; six strains of his groups III A or III B were our subgroup 3. Three other strains of our subgroup 3 were intermediates in Gibson's scheme; two strains were II–III and the third (NCTC 963) a I–II intermediate. Non-fermenting strains form group II of Gibson's classification, and explain why our subgroup 3 seems to show a spread over his groups. We think our arrangement is the more logical; the subgroups 1–5 show a gradual transition from the biochemically active strains of subgroup 1 to the inactive strains of subgroup 5. The results support our original hypothesis that these cocci can be arranged in some order so that their reactions show all the gradations of a spectrum.

Subgroup 2 seems to be the coagulase-negative counterpart of subgroup 1. Between subgroups 2 and 3 there are significant differences; strains of subgroup 2 grow best at 37°, produce acetoin, seldom ferment xylose or salicin, and seldom produce lemon-yellow pigments. Subgroup 3 is more often characterized by a lower optimal temperature, a higher proportion of strains ferment xylose and salicin, and more strains produce yellow pigment. Subgroup 4, the glucose-fermenting strains that produce a pink pigment, seem to form a link between subgroup 3 and the inactive subgroup 5.

Can each of these subgroups (2–5) be given specific status? Mannitol fermentation was used in the classification of these cocci by Andrewes & Gordon (1907), Dudgeon (1908), Julianelle (1937), Chapman, Lieb & Curcio (1938), and by others; it was not considered of value by Winslow & Winslow (1908), or by Winslow, Rothberg & Parsons (1920). With glucose, it appears to be one of the most constant of the fermentation reactions (Blair, 1938) and these have the greatest claim to differential value of all the 'sugar' tests. We accept glucose fermentation as a differential test; but mannitol fermentation seems to have more limited value.

Pigmentation has long been the main differential character in this genus. We found that the pigmentation of strains in subgroups 3–5 remained constant in cultures maintained on media for many years; pigmented strains of groups 1–2, however, continually threw off stable non-pigmented (white) variants; the pigmented form could only be retained by careful colony selection and maintenance of the culture in dried form. It is seldom possible to classify old strains of these groups by pigmentation, and, as freshly-isolated pathogens may be devoid of pigment, we think this criterion should be used only for the differentiation of the relatively inactive strains of subgroups 3–5. The pink pigment producers of subgroup 4 are remarkably constant, and in this group we have never observed a non-pigmented variant. On the other hand, the pink strains in subgroup 5 were less constant in pigmentation.

NOMENCLATURE

With the exception of the α-group our series is regarded as being of one genus. The genus as we shall define it is a combination of species of several genera; it includes species of *Sarcina*, *Micrococcus*, *Staphylococcus*, *Gaffkya* and *Rhodococcus*, but excludes the strictly anaerobic species. The Winslows (1908, pp. 104, 106) retained the genus *Sarcina* because the name was 'firmly intrenched in the literature and usage of bacteriology' (p. 228), although they admitted that packet formation was an inconstant character. We would restrict the generic name *Sarcina* to the strictly anaerobic Gram-positive cocci. The characterization of the genus *Micrococcus* Cohn, 1872, was based on several different types of micro-organism, including species now known by the names *Chromobacterium prodigiosum*, *Chrom. violaceum* and *Pseudomonas pyocyanea*. Rule 24 (7) of the Bacteriological Code (Buchanan, St John-Brooks & Breed, 1948) states that the name of a taxonomic group (taxon) is illegitimate if 'the characterization of the group was based upon an impure or mixed culture'. We do not think that this rule is abused if it is interpreted to make the generic name *Micrococcus* Cohn illegitimate on the grounds that the characterization was based on cultures of different types. A type species was not chosen by Cohn but the first-named species, *M. prodigiosus*, implies that if the generic name is valid it should replace the name *Chromobacterium*. Although we have the weight of Buchanan's (1915) opinion against us, we reject the generic name *Micrococcus* in the belief that our interpretation of Rule 24 (7) is correct. The name *Staphylococcus* was used by Ogston (1882), who clearly differentiated the pigmented cluster-forming cocci from the streptococci. Rosenbach (1884, p. 18) accepted Ogston's name and adopted it for a group of two provisional types (für eine Gruppe von vorläufig 2 Einzelarten) which he proposed to name, according to the botanical system, *Staphylococcus flavus* or *aureus* and *Staph. albus*. He described these species under the trinomials *Staph. pyogenes aureus* (p. 19) and *Staph. pyogenes albus* (p. 21), but later (p. 27) reverted to the binomial *Staph. aureus*.

Our subgroup 1 can be identified as *Staph. aureus* Rosenbach and subgroup 4 as *Micrococcus roseus*. Fairbrother (1940) used the name *Staph. saprophyticus* for coagulase-negative strains of human and animal origin and, although he said they were usually non-fermenters of mannitol, we suggest that this is a suitable name for our subgroup 2. It has the advantage that it avoids a name descriptive of a pigment.

Subgroup 3 probably includes several previously described species; rule 19 of the Code requires that the oldest legitimate epithet should be retained. Abd-el-Malik & Gibson (1948) have indicated the confusion attached to such names as *M. luteus* and *M. varians*, and we think that further confusion will arise if we attach to our subgroups names that are in current use for inadequately characterized species. Subgroup 3 we designate as *Staph. lactis* n.sp. Subgroup 4 strains form the pink pigmented species; the Winslows (1908) gave this group generic rank but apart from pigment the group forms a natural connecting link between subgroups 3 and 5. Ten of our strains were labelled *M. roseus* and we see no reason for changing the specific epithet.

The non-fermenting, biochemically inactive strains of subgroup 5 are common in nature but, because of inadequate characterization in the earlier literature, they are difficult to name. Castellani once sent a culture (now dead) labelled *Staph. afermentans* to the NCTC but we have not found this name in the literature. We think it is an appropriate name and use it for subgroup 5, ascribing it to Castellani.

DEFINITIONS

To give greater precision to our definitions each is divided into two parts, essential characters, and usual or variable characters. A type culture of *Staph. aureus* Rosenbach has never been selected; we have designated type cultures of this species and of the others we have defined, together with the corresponding catalogue numbers in this Collection (Rule 9, Recommendation 9*d*).

Staphylococcus Rosenbach

Essential characters. Gram-positive spheres in pairs or irregular clusters. Catalase-positive. Aerobic and facultatively anaerobic; never strictly anaerobic. Not acid-fast. Gas never produced from the breakdown of carbohydrates.

Usual and variable characters. Usually non-motile and non-sporing. Many strains are active fermenters of carbohydrates, some are proteolytic. Most strains grow in medium containing 10 % NaCl.

Type species. Staphylococcus aureus Rosenbach emend.

Species (i). Staphylococcus aureus Rosenbach emend.

Essential characters of the genus. Coagulase-positive (human or rabbit plasma). Acid produced from glucose.

Usual characters. Colonies usually coloured golden-yellow or cream; white colonies occur frequently as variants and may be the only type present in a culture. Lemon-yellow strains are rare. Usually produce acetoin, reduce nitrates and methylene blue, and liquefy gelatin. Usually hydrolyse urea and acidify milk.

Type strain. FDA 209-P (ATCC 7538P, NCTC 7447).

Species (ii). Staphylococcus saprophyticus Fairbrother emend.

Essential characters of the genus. Coagulase-negative. Acid and acetoin produced from glucose.

Usual characters. Colonies usually not pigmented. Usually reduce nitrates and methylene blue, hydrolyse urea, liquefy gelatin and acidify milk.

Type strain. S 41 (NCTC 7292).

Species (iii). Staphylococcus lactis n.sp.

Essential characters of the genus. Coagulase-negative. Acid produced from glucose. Acetoin not produced. Colonies not pink.

Usual characters. Cells often arranged in tetrads or packets. Usually reduce nitrates and methylene blue. Liquefaction of gelatin and hydrolysis of urea variable.

Type strain. G 33 (NCTC 7564)

Species (*iv*). *Staphylococcus roseus* Tavel.

Essential characters of the genus. Coagulase-negative. Produce acid from glucose; acetoin not produced. Produce pink colonies on agar.

Usual characters. Usually reduce nitrates but not methylene blue. Usually do not hydrolyse urea, liquefy gelatin, or acidify milk.

Type strain. ATCC 418 (NCTC 7520).

Species (*v*). *Staphylococcus afermentans* ex Castellani.

Essential characters of the genus. Coagulase-negative. Do not produce acid or acetoin from glucose.

Usual characters. Usually do not reduce nitrates or hydrolyse urea. Methylene blue reduced. Gelatin usually liquefied.

Type strain. NCTC 2665 (*Micrococcus lysodeikticus* Fleming).

We wish to acknowledge our indebtedness to the many workers who sent us strains, and especially to Dr Ruth Gordon and Dr T. Gibson for their co-operation. Dr Gibson's knowledge of the older literature was invaluable in dealing with the problems of nomenclature. Most of the work was done at the Lister Institute of Preventive Medicine, Elstree, and we are grateful to the long-suffering staff of the media room there.

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The L-form of *Haemophilus pertussis*

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SUMMARY: Ageing, chilling, and media containing suitable concentrations of glycine were found to stimulate the production of L-forms in cultures of *Haemophilus pertussis*. Stained with Giemsa these L-forms are morphologically similar to the L-forms of many other bacilli produced by similar stimuli. Serologically the L- and bacillary forms appear to be related. The suggestion is put forward that the small round red form is a 'resistant' form of *H. pertussis* and reverts to the rod by passing through the stages characteristic of the L-cycle.

Information on the structure and morphological variation of *Haemophilus pertussis* is very limited when compared with that of many other Gram-negative species. One of the interesting attributes of these other species is their ability to produce large bodies or L-forms (according to the terminology of Klieneberger, 1935) when subjected to suitable stimuli. This investigation was undertaken, therefore, to determine whether *H. pertussis* was similar to other Gram-negative bacilli in this morphological feature.

MATERIALS AND METHODS

Strains. Freshly isolated and old laboratory strains of *H. pertussis* were used. They included NCTC 364, one of Leslie & Gardner (1931) Phase IV strains; 18–323, Kendrick, Eldering, Dixon & Misner (1947) challenge strain; and 29, the Lederle Laboratories toxin-producing strain.

Media. Cultures were grown on Bordet & Gengou (BG) medium containing 30 % horse blood. For mass production of the pure L-form, cultures were grown on BG containing 1·5 % (w/v) glycine. Unless otherwise stated, incubation was at 35° – 36°.

Preparation of slides. At appropriate times during incubation 6 small squares were cut from the plate cultures and placed culture-side down on coverslips. The agar was then flicked off with the tip of a scalpel, and the coverslip film fixed in osmium tetroxide vapour for 4 min. Three were stained in 3 % aqueous solution of Giemsa (R66, Gurr, London) for 3 hr. Three kinds of organisms, blue, red and purple, could be differentiated by this method.

For demonstration of nuclear structure the other three coverslip films were treated with N-HCl for 3 min. at 55° before staining with Giemsa.

Preparations were dehydrated in acetone-xylene mixtures and mounted in Canada balsam (Klieneberger-Nobel, 1950).

Standardization of suspensions. The density of bacillary suspensions was estimated by comparison with Brown's Opacity Tubes (Burroughs Wellcome and Co. Ltd., London) assuming that *H. pertussis* was equivalent to *H. influenzae*. Because of variation in size and shape of L-forms, no really satisfactory method for estimating the density of L-suspensions has been found; in these experiments the L-suspensions were adjusted by the same method used for bacillary suspensions. Preliminary N determinations indicated that L-suspensions equivalent by opacity to bacillary suspensions might contain twice as much N.

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Immunization. Rabbits were injected intravenously at weekly intervals with suspensions of living bacilli (25×10^8/ml.) in two doses of 0·5 ml. followed by four doses of 1·0 ml. When L-suspensions were administered the last four doses contained formolized organisms.

Agglutination tests. Serial twofold dilutions from 1/200 to 1/25,000 were made of anti-L or anti-bacillus serum. Antigens were living L-forms or bacillary forms suspended in Bacto-Casamino acids (Baird and Tatlock, Ltd., London) or formolized suspensions of L-forms in saline. Serum and antigen were mixed in 0·5 ml. amounts, incubated at 56° for 4 hr., and read after standing overnight at room temperature.

EXPERIMENTAL

Effect of ageing

A 24 hr. culture of a smooth strain of *H. pertussis* contained short blue rods and an occasional small round red form. The culture was set aside at room temperature for 4 weeks; the medium was then dried and cracked, the blood haemolysed and blackened, and the growth sunken into the agar and difficult to remove with a platinum loop. The organisms were then all in the small round red form; rarely, minute red granules or very pleomorphic red rods were observed, but no blue organisms were found. The culture was transferred to fresh BG medium, incubated and examined after various times. The following changes were observed.

After 15 *hr.* Some of the round red forms had become larger, others had become paler and no longer had well-defined outlines. Some blue granules and a few small blue pleomorphic rods had appeared. The great variation in staining intensity and in the shape of the organisms was the most notable feature.

After 21 *hr.* Red round forms were usually large, and large round purple bodies had appeared. The background showed a pale red haze. Blue rods were numerous and usually larger than ordinary size.

After 48 *hr.* The microscopic colonies which had appeared were composed of clumps of large red and purple bodies surrounded by red, purple, and blue rods. There was a dense red background haze and many granules in and around the clumps. When the culture was serially passaged on fresh BG medium the organisms reverted to ordinary *H. pertussis* bacilli. A schematic representation of the sequence of the various morphological forms is shown in Fig. 1.

Other cultures, both smooth and rough strains, aged in the incubator for 8 days underwent similar changes. All old cultures composed of the small round red forms were viable, and all reverted to ordinary bacilli after several passages on BG medium, at 48 hr. intervals.

It is well known that old cultures often contain extremely pleomorphic forms, but it has not been proved that these are related to L-forms. Nevertheless, the alterations that took place were suggestive of the L-cycles described for other bacilli (Klieneberger-Nobel, 1949).

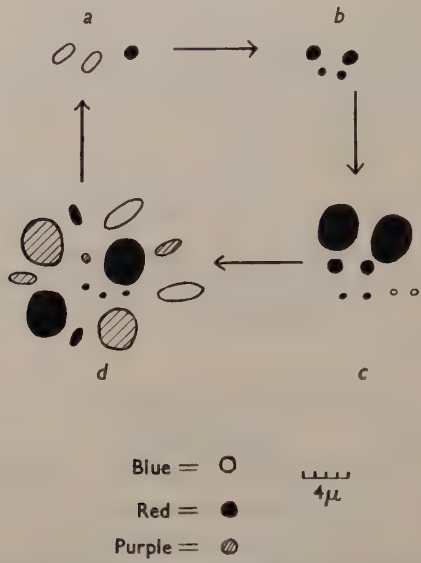

Fig. 1. Diagram of morphological changes in *H. pertussis* on BG medium. (*a*) Parent culture at 24 hr.; and subculture II at 24 hr. (*b*) Parent culture at 4 weeks. (*c*) Subculture I at 15 hr. (*d*) Subculture I at 48 hr.

Effect of chilling

One stimulus known to induce the L-cycle is interruption of growth by a period of refrigeration (Klieneberger-Nobel, 1949). An overnight culture was transferred to fresh BG medium, incubated for 5 hr., stored at 2–4° for 18 hr., and returned to the incubator for 24 hr. The morphological findings were as follows.

After 5 *hr. at* 35°. Mostly purple rods, often in pairs; some blue rods and a very few small round red forms.

After 18 *hr. at* 2–4°. Mostly blue rods; some purple rods; fairly numerous small round red forms.

After 7 *hr. at* 35° (*second incubation*). About half the organisms were in the round red form, either small or medium-sized; the rest were blue rods. Some red granules were present, and a red haze coloured the background.

After 24 *hr. at* 35°. Mostly blue rods and some small round red forms (as in Fig. 1*a*).

Although the size and relative proportion of red forms that developed varied from strain to strain, all strains tested exhibited the transformation. Rough strains, especially no. 364, yielded much larger bodies, and a greater number of bodies than smooth strains. As little as 1 hr. at 2–4° produced a perceptible increase in red forms, but it was necessary to chill during a period of rapid multiplication to produce a pronounced transformation to the round red form.

From this experiment it was apparent (*a*) that *H. pertussis* reacted to chilling by producing several forms morphologically similar to the L-forms of other bacilli; (*b*) that when stimulated *H. pertussis* underwent a series of transformations similar to known L-cycles before returning to bacillary form; (*c*) that the forms induced by chilling were similar to those that appeared in old cultures.

Effect of glycine

L-transformation can be induced by growth in the presence of glycine (Gordon & Gordon, 1943; Dienes, Weinberger & Madoff, 1950). A 24 hr. culture of a smooth strain was planted on BG medium containing 0·75 % (w/v) glycine and incubated at 30°. At the time of the transfer (0 hr.) and at hourly intervals thereafter impression preparations of the growth were examined. The changes that occurred were as follows.

At 0 *hr.* Mainly blue rods, some small red round or rod-shaped forms.

After 1 *hr.* Blue rods, fatter; more red organisms, some triangular or irregular in shape (Pl. 1, fig. 1).

After 3 *hr.* Blue organisms very fat and pale, often showing red granules central or at each pole. Red organisms comprising *c.* 50 % of the total, had become larger and their pleomorphism had increased (Pl. 1, fig. 2).

After 4 *hr.* Further increase in size and number of red organisms with small red free-lying granules.

After 5 *hr.* Almost no blue organisms. Red organisms of three sorts: (*a*) large bodies of various shapes, (*b*) slim long rods with a granule or thickening at centre, (*c*) small free-lying granules. Red haze in background prominent (Pl. 1, fig. 3).

After 7 *hr.* Red bodies mottled, unevenly stained, boundaries ill-defined.

After 9 *hr.* Mottling increased causing bodies to appear granulated; most intense granulation often stained purple.

After 10 *hr.* The bodies appeared to be dividing into numerous segments, each segment containing a portion of the darkly stained granular material. The granular material had often coalesced into a dense lump, and much more of it was now stained purple (Pl. 1, fig. 4).

After 26 *hr.* Many bodies breaking down into fragments stained red or purple, and scattered in a very dense red background haze (Pl. 1, fig. 5).

After 4–5 *days.* All large bodies had disappeared. Only red granular material and some small round red forms remained (similar to Fig. 1*b*); background haze dense and full of red amorphous material.

When the 26 hr. culture was transferred to fresh BG medium + glycine and passed serially at 24 hr. intervals in glycine medium, a few bacillary forms appeared at the second or third transfer. They disappeared, however, on the next transfer, and by the fifth or sixth passage the culture looked the same as the 4–5 days' culture above. It became impossible to continue passage of these cultures because of the sparsity of growth.

When the 4-day culture was transferred to fresh BG medium without glycine, growth occurred and the organisms gradually reverted to rods. Cultures could be maintained indefinitely in daily serial transfer, using alternately ordinary BG medium and BG medium + 1·5 % glycine, or could be carried indefinitely on BG medium + 0·375 % glycine without alternate transfers on glycine-free medium.

The nuclear structure of the various forms was also examined (Pl. 2, fig. 1). The large bodies contained chromatinic granules scattered throughout, arranged evenly around the circumference or arranged in clumps and networks. The small round form had a single, central chromatinic granule. The granular forms appeared to be composed wholly of chromatinic material. The nuclear structure in each case was similar to that seen in the L-forms of other bacterial species.

The order and rate of the alterations described differed in detail for different strains and with any change in the conditions of the experiment. The size of the bodies formed varied greatly from strain to strain. A rough strain (no. 364) regularly showed occasional small round red forms *c.* 1·0 μ. diameter in all 24 hr. cultures on BG medium. When such cultures were transferred to BG medium + 0·75–1·5 % glycine, the organisms were completely transformed in 12–18 hr. into large bodies, many of which were the size of a horse erythrocyte (Pl. 2, fig. 2). Cultures of strains freshly or quite recently isolated from cases of whooping-cough produced small bodies up to 3 μ. diameter when grown on glycine-containing medium.

The concentration of glycine influenced the size of the bodies produced and the completeness of the transformation. A concentration of 1·5 % glycine produced complete transformation to the L-form in 24–30 hr. in all thirty-five strains investigated, whereas 0·75 % glycine gave more variable results, especially with recently isolated strains.

The temperature of incubation affected the rate of transformation; at 30° it was usually easier to observe the various stages in the transformation toward large bodies than at 35°, when the changes took place more rapidly and with even less uniformity.

The shapes assumed by an organism during transformation are probably determined mainly by physical factors. The rigidity of the cell wall seemed to diminish and the organism to become plastic; its shape in a colony appeared to be determined by the space allowed by the surrounding organisms, so that the individuals of a colony fitted together in a mosaic. In spite of this, some strains regularly formed angular and many-pointed bodies, whereas other strains formed oval or rounded bodies.

The age and condition of the parent culture at the time it was placed on glycine medium were reflected in the subsequent changes that took place. Parent cultures (5, 24 and 96 hr. old), grown on BG medium, were all transformed into large bodies after incubation for 24 hr., but the early stages of the transformation followed slightly different patterns in each case. It seemed as if the previous history of each individual organism in a culture determined to some extent the sequence followed when it was placed on glycine medium, and for this reason individuals may be found in the same culture in different stages in the transformation toward the large bodies. The type of changes that took place and the manner in which they occurred when *H. pertussis* was grown in the presence of glycine left little doubt that we were dealing with true L-forms. In its ability to undergo the L-transformation, *H. pertussis* was, therefore, similar to other Gram-negative bacilli.

Effect of arginine and peptone

Arginine monohydrochloride (1 %, w/v) caused transformation to the L-form in five of thirty-five strains and a slight increase in L-forms in a few more. Only smooth cultures were thus stimulated by arginine.

Peptone (Oxoid) at 2 % (w/v) induced L-formation in all the more recently isolated strains but did not affect rough or old but still smooth laboratory strains.

Cultural characteristics

The appearance of the growth of *H. pertussis* in its L-form had certain characteristics distinct from the bacillary form of growth. It grew on the surface of the BG agar in a thin, flat film, so delicate, that at first glance its presence was scarcely noticed. The centre of the colony grew down into the agar and this habit may account in part for the great tenacity of the culture to the agar. Individual colonies could usually be observed during the first 24 hr. or longer only under a hand lens or the low power of the microscope. To study the colony form under the microscope, it was necessary to add haemolysed blood instead of whole blood to BG medium to make the medium sufficiently transparent. Colonies had a dark centre and a lacy granular periphery. This structure was similar to the structure of L-colonies of *Streptobacillus moniliformis* (Klieneberger, 1938). The L-growth of *H. pertussis* produced bubbles and droplets of a fluid which has not yet been identified and which was usually overlooked in stained preparations, but prominent by phase contrast. The L-bodies rested on the circumference of these droplets and appeared as dark crescent-shaped caps (Pl. 2, fig. 3). The formation of bubbles of fluid also has its counterpart in *Strep. moniliformis* which forms droplets of cholesterol during its growth in the L-phase (Partridge & Klieneberger, 1941).

Serology

A close serological relationship has been found to exist between bacillary and L-forms of some organisms (Klieneberger, 1942; Weinberger, Madoff & Dienes, 1950). The serology of the two forms of *H. pertussis* is still under investigation; results obtained so far indicate the existence of a relationship between them. As shown in Table 1, the sera produced with either L- or

Table 1. *Agglutination of L- and bacillary forms of* Haemophilus pertussis

Serum	Antigen	Titre
Anti-L	L (formol)	1/3200
	Bacillus (living)	1/3200
	L (living)	0
Anti-bacillus	L (formol)	1/6400
	Bacillus (living)	1/6400
	L (living)	0

bacillary cultures reacted equally well with suspensions of living bacilli or formolized L-forms, but neither serum when diluted reacted with the living L-antigen. The poor agglutinability of the living L-form is not understood, but even the bacillary form of certain strains of *H. pertussis* is not agglutinated in the absence of formaldehyde (Robbins & Pillemer, 1950).

DISCUSSION

From stained preparations it was apparent that *H. pertussis*, under appropriate stimulation, changed into the various forms characteristic of the L-cycle and from these back to rods, but the order in which these changes occurred could not be reconstructed from the fixed preparations.

The significance of the small round red form is not clear; it is not known whether it corresponds to the coccoid form found in ordinary Gram-stained films nor whether it can be distinguished from other coccoid forms when observed by phase-contrast microscopy. It has been found in practically all ordinary cultures of *H. pertussis* which, so far as is known, are not under the influence of special stimuli. It is least numerous in 5–12 hr. cultures on BG medium and almost the only form present in very old cultures. It appears regularly in abundance during some stage of the L-cycle however the transformation is induced; therefore, it seems possible that *H. pertussis* may regularly employ two methods of reproduction: (*a*) simple binary fission; (*b*) multiplication *via* the L-cycle.

The small round red form may be a resistant form of *H. pertussis*, resistant to certain highly unfavourable environmental conditions which affect all or few organisms in a culture. This could account for the marked increase in the small red forms when cultures were subjected to chilling or prolonged ageing. When the red forms were moved to an environment suitable for growth and multiplication, they reverted to the bacillary form, and during this reversion the various forms typical of the L-cycle appeared. When the organisms were allowed to remain in an environment unsuitable for their multiplication, as in the presence of high concentrations of glycine, the rods disappeared first; the last forms to disappear were the granular and small round red forms. The study of living preparations will probably be one of the best means of acquiring the information necessary to understand the significance of the L-form.

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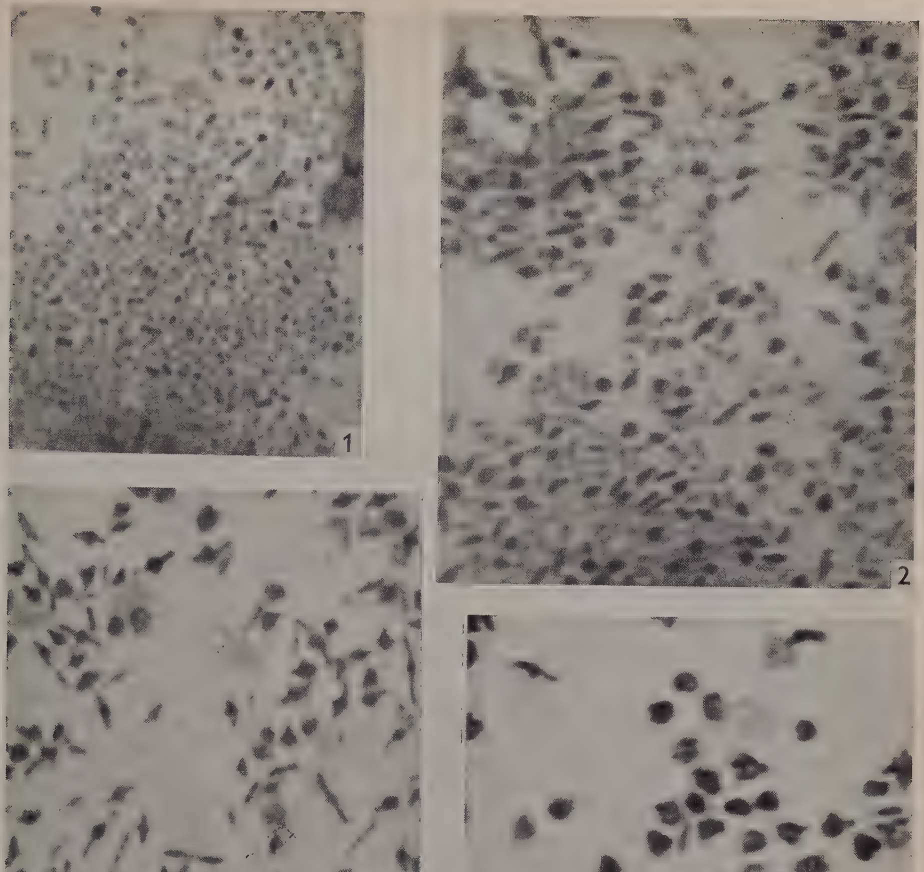
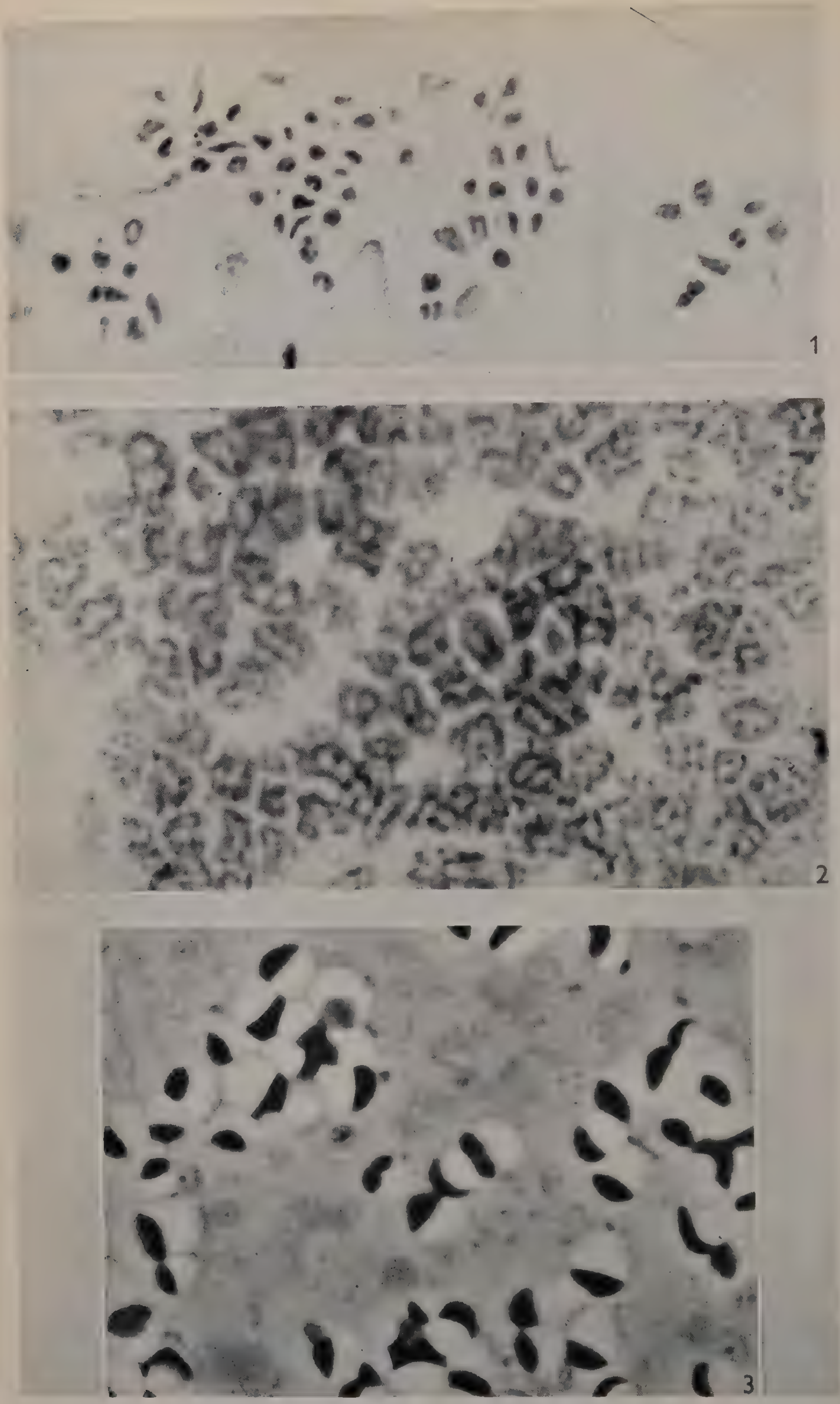


Figs. 1–5

R. G. WITTLER—THE L-FORM *HAEMOPHILUS PERTUSSIS*. PLATE 1



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EXPLANATION OF PLATES

Plate 1

Osmium tetroxide Giemsa impression preparations of *H. pertussis*, strain no. 18–323, grown on BG medium containing 0·75 % (w/v) glycine; incubated at 30°; × 3500.

Fig. 1. After 1 hr. incubation. Blue organisms show up in the photograph as grey and red ones as black; a few pleomorphic forms have appeared.

Fig. 2. After 3 hr. incubation. Blue organisms as grey and red ones as black; size of organisms increased and a larger proportion of red ones; pleomorphism very marked.

Fig. 3. After 5 hr. incubation. Red organisms show as black, and red background haze and pale red organisms as grey; no blue organisms; all organisms in some stage of the L-cycle.

Fig. 4. After 10 hr. incubation. In the photograph the purple material cannot be distinguished from the red in the large bodies; the red background haze which appears grey is widespread; some large bodies are starting to segment.

Fig. 5. After 26 hr. incubation. Edge of colony showing fading and fragmentation of large bodies. Remnants of bodies are often difficult to distinguish from the red background haze.

Plate 2

H. pertussis, × 3500.

Fig. 1. Strain nos. 18–323; osmium tetroxide HCl Giemsa preparation showing nuclear structure of L forms after 9 hr. incubation at 30° on BG medium + 0·75 % (w/v) glycine.

Fig. 2. Strain no. 364; osmium tetroxide Giemsa preparation; 19 hr. growth at 36° on BG medium + 1·5 % (w/v) glycine. Fragmentation of large L bodies.

Fig. 3. Strain no. 364; phase-contrast; 24 hr. growth at 30° on serum infusion + 1·5 % (w/v) glycine agar. Organisms in form of dark caps at circumference of clear drops of fluid.

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[The Editors of the Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.]*

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Eleventh General Meeting at Bristol University on Tuesday and Wednesday, 19 *and* 20 *September* 1950. *The following communications were made:*

COMMUNICATIONS

Osmophilic Yeasts in Raw Beet and Cane Sugars and Intermediate Sugar Refining Products. By M. Pamela Scarr (*Tate and Lyle Ltd., Keston*)

Osmophilic yeasts are found in the film of molasses on the crystals of raw sugars and in intermediate sugar refining products of a wide range of concentration. They are allied to yeasts found in similar situations such as concentrated fruit juices, etc., and can be made to spore more or less freely under dry conditions, usually with conjugation. They are unable to destroy sucrose in strongly buffered solutions, but in lightly buffered solutions at pH values *c.* 5 some are able to produce sufficient acid from traces of invert present to gradually invert the sucrose. During this process multiplication takes place. Previous workers have already shown that the majority of this group are highly acid resistant.

Organisms which grow in high concentrations of dissolved solids apparently show an adaptive ability at acid pH values only, which is lost after a period of 6·8 weeks in dilute media but can be revived in many cases by increasing the sugar concentration in not less than two. Temperature resistance is also increased in concentrated media.

Concentrated syrups tend to depress the tendency to form pseudo-mycelium if any, the cells also being slightly smaller.

Observations on the Growth Requirements of Organisms of the Pleuropneumonia Group. By D. G. ff. Edward and W. A. Fitzgerald (*Wellcome Veterinary Research Station, Frant*)

An etherial extract of egg-yolk could replace serum in culture media. Fractionation suggested that cholesterol might be the active substance. When pure cholesterol alone was used to replace serum only, occasional poor growth resulted. Better results were obtained when starch or serum-protein was added with the cholesterol, but colonies were slow in appearing. More rapid growth, as good as with serum, resulted from the addition of cholesterol together with the acetone-insoluble fraction from egg-yolk. Purified preparations of lecithin and kephalin, prepared from the acetone-insoluble material, did not possess this growth-promoting property. Since starch, serum protein and acetone-insoluble lipoid could not themselves alone replace serum, cholesterol appeared to be an essential growth factor; it was most effective at a concentration of 10 mg./100 ml. of medium.

There appear also to be other factors concerned in growth. For instance, it was shown that growth of a strain of *Asterococcus bovis* in media enriched with horse serum was improved by the addition of a small amount of oleic acid; larger amounts of oleic acid were inhibitory for all strains.

Penicillinase Adaptation in *Bacillus cereus*. By M. R. Pollock (*National Institute for Medical Research, Mill Hill*)

The penicillinase of *B. cereus* hydrolyses penicillin to penicilloic acid and its production has been followed manometrically by the technique of Henry & Housewright (1947). Its formation is stimulated up to 30-fold by the presence of penicillin, and, like many other bacterial enzyme adaptations, will occur in washed cell suspensions without added N and in the absence of growth. The adaptation is stimulated by glucose, O_2 and added amino-acids, and inhibited by oxine. Very small concentrations of penicillin (e.g. 0·04 unit/ml.) are sufficient to cause marked adaptation which is highly specific. It is, in fact, only necessary to treat cells with penicillin for up to 1 hr. at 0° (followed by thorough washing to remove all free penicillin) to enable them to form penicillinase rapidly when subsequently incubated in a penicillin-free medium. The production of the enzyme, after this pretreatment, proceeds at a constant rate and will so continue apparently indefinitely so long as the medium is replenished, although the cells themselves are growing logarithmically. The activity per cell thus gradually falls to the normal, unadapted level, over a period covering several cell generations.

The rate of penicillinase formation following pretreatment increases with increase in concentration of penicillin used up to 1 unit/ml., at which level maximal effect occurs.

The results are consistent with the hypothesis that penicillin reacts with some sort of receptor within the cells to form a complex or compound which acts as a specific catalyst stimulating penicillinase formation.

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The Synthesis of Serine by Lactobacilli and Other Micro-organisms. By June Lascelles and D. D. Woods (*Department of Biochemistry, University of Oxford*)

In growth experiments with *Leuconostoc mesenteroides* P-60 the requirement for serine was found to be replaced by glycine (*c.* 10^{-2} M) provided that (*a*) pyridoxal and *p*-aminobenzoate (*p*-AB) were present, and (*b*) that the cultures were incubated in an atmosphere enriched with CO_2. Pyridoxal and CO_2 were essential while without *p*-AB, growth was reduced to a half. Citrate, oxaloacetate and α-ketoglutarate, though not formate, partially replaced CO_2. These observations made possible an assay for serine in the presence of excess glycine.

Suspensions of *Streptococcus faecalis* R deficient in the vitamin B_6 group and folic acid synthesized serine in a system containing only phosphate buffer, glucose, glycine, formate, pteroylglutamate (PtG) and pyridoxal. Only traces of serine were found when either glycine, formate or glucose were omitted. PtG was essential and pyridoxal highly stimulatory. Formyl-PtG was consistently rather more active than PtG, rhizopterin (with or without glutamate) less active, whilst *p*-AB and vitamin B_{12} were inactive.

Similar experiments with folic acid-deficient cell suspensions of a strain of *Saccharomyces cerivisiae* (requiring *p*-AB for growth) showed that the addition of *p*-AB doubled the synthesis of serine; PtG, which does not replace *p*-AB for the growth of this organism, was inactive.

Plaut, Betheil & Lardy (1950) have found recently that rats deficient in folic acid incorporated less carbon from $H.C^{14}OOH$ into tissue serine than did the control animals.

These results, both with growing organisms and cell suspensions, support current suggestions that folic acid derivatives may be concerned in reactions involving the incorporation of one-carbon fragments.

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Relative Availabilities of Various Soluble Carbohydrates for Starch Production by Ciliated Protozoa of the Sheep's Rumen. By Frances M. Masson and A. E. Oxford. (*Rowett Research Institute, Bucksburn*)

Of all the soluble carbohydrates tested only glucose, fructose, sucrose, inulin, bacterial levan, and to a lesser extent cellobiose, are converted into 'starch' by relatively bacteria-free washed holotrich ciliates isolated from the rumen contents of hay-fed sheep. By 'starch' in this connexion is meant a glucosan, stored in the form of granules inside the protozoa, which gives a purple colour with iodine due to the formation of a complex which is precipitated in presence of 6% NaCl. The protozoan starch yield so obtained may be considerable and a 50 g. specimen has been prepared by use of the withdrawn rumen contents from two fistulated sheep over a number of weeks. In presence of glucosamine hydrochloride (0·3%) the holotrich ciliates are rapidly destroyed leaving the oligotrich ciliates intact. A washed suspension of the latter also yields a 'starch' giving a purple colour with iodine. This starch disappears when the washed oligotrichs are incubated overnight at 40° in the presence of toluene. The oligotrich ciliates do not appear to convert cellobiose into starch.

Haemophilus pertussis as an Homologous Bacterial Type. By J. Ungar and W. K. Stevens (*Glaxo Laboratories Ltd., Greenford*)

Of the 208 strains which were examined, 187 were freshly isolated and found 'virulent' to mice, and 10, kept in the laboratory, were 'avirulent' to mice; 11 *parapertussis* strains were also tested. Each strain was subjected to the following examination:

(1) Agglutination with immune sera against virulent, avirulent and parapertussis strains.

(2) Virulence test in mice by measuring the LD 50 after intranasal infection.

(3) Toxicity to mice after intraperitoneal, and to rabbits after intradermal injection.

(4) Morphological examination of the culture (macroscopically and microscopically).

(5) Haemagglutination of a 2 % suspension of human red cells.

(6) Growth requirements on solid and fluid media.

(7) Cross-absorption tests of agglutinins.

Freshly isolated strains of *H. pertussis* form a single antigenic type in contrast to the avirulent and *H. parapertussis* strains, though all have a common antigen. Haemagglutination was observed with the virulent *H. pertussis* and *H. parapertussis* but not with avirulent strains. Virulent strains grew on solid media only in presence of blood, and in fluid media only after addition of starch. These strains were completely precipitated by a 2 % suspension of aluminium phosphate; avirulent strains are precipitated only partly or not at all.

Toxicity to mice or rabbits is not a criterion for differentiating between the three types. Avirulent variants induced by growing virulent strains on unfavourable media are irreversible and antigenically distinct from the virulent form. In immunological studies the virulent and avirulent strains form two separate types, easily distinguished by the tests cited above.

The Role of Zinc in Metabolism. By G. N. Rolinson (*Boots Pure Drug Co. Ltd., Nottingham*)

Zinc is essential for the growth of all living organisms, but almost nothing is known of the precise role of the metal in metabolism.

It has been generally concluded that the element plays a part in the utilization of carbohydrate by fungi, because under conditions of partial zinc deficiency less carbohydrate is completely oxidized and a greater proportion is diverted to form organic acids.

The results of experiments with *Aspergillus niger* confirm this effect of partial zinc deficiency, but show also that the effect is by no means specific for zinc. Partial deficiencies of iron, copper, manganese, magnesium, phosphorus and potassium, all have the same effect on acid production, and it can no longer be accepted that zinc plays a specific function in carbohydrate metabolism.

The Influence of Proteins and Polypeptides in Growth Media upon the Antigenic Structure of *Erysipelothrix rhusiopathiae*. By A. W. Gledhill (*National Institute for Medical Research, Mill Hill*)

New Methods of Assay for the Antibiotic Nisin. By R. Friedmann and S. A. Beach (*Fisons Ltd., Loughborough*)

The isolation, properties and the potential uses for nisin in the Medical and Veterinary field have been described (Mattick & Hirsch, 1947, 1949) and investigations have been in progress in these laboratories for some time to develop these findings. Several methods of assay have been examined, and two methods are described here which have advantages over those previously published, particularly for process development, where assays on a large number of samples are required.

It was thought at one time that nisin could not be made to diffuse through agar (Mattick & Hirsch, 1947, 1949) or only with great difficulty (Hirsch, 1950). Nevertheless, it has now been possible to devise a diffusion method by adding Crill 20 to the medium, a device similar to that used by Stansly & Schlosser (1947) for polymyxin. Diffusion was allowed to proceed overnight in the refrigerator before allowing the test organism (*Streptococcus agalactiae*) to commence growing. A tube method was preferred to the original cup plate methods. Straight-line relationships hold good between 50 and 2000 units/ml. Five-point assays give standard errors of 15–20 % and the method is so simple that many more repeats can readily be performed. Drawbacks are the long duration of the test (48 hr.) and that impurities, particularly as contained in crude extracts, may interfere.

To overcome such difficulties encountered previously also with the other methods, a reductase method was evolved based on a rough methylene-blue test described by Hirsch (1950): the same fast-growing strain of *Strep. cremoris* was employed, but methylene blue was replaced by resazurin and a procedure devised for obtaining reproducible inocula in the correct phase of growth. It will be shown that there is no action whatever on organisms in the lag-phase, but due to the extremely rapid bactericidal action of nisin in the log phase, results are obtainable within 20 min. The standard error for five estimations is of the order of 10 %. Interference from other constituents and even selected organic solvents has not been encountered. The test has proved suitable for the assay of nisin in body fluids.

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The Morphology and Life Histories of the Organisms Inhabiting the Root Nodules of Certain Non-leguminous Plants. By LILIAN E. HAWKER and J. FRAYMOUTH (*Botany Department, Bristol University*)

Root-nodules of *Elaeagnus* spp., *Hippophae rhamnoides, Alnus Glutinosa* and *Myrica gale* which contain an endophyte, were described. The enlarged cortical cells of an actively growing nodule contain the endophyte in several stages of development. The apical meristem is free from infection but the cells just behind it show a plasmodial stage. Further back from the apex, spherical vesicles in species of *Elaeagnus, Hippophae* and *Alnus* and club-shaped bodies in *Myrica* are formed and from these small, 'bacteroid'-like granules are produced. There is some evidence that granules formed in different host cells fuse in pairs and possess some power of migration within the nodule. No definite hyphae can be seen in any of the nodules but well-marked protoplasmic strands or lines of flow are present, particularly in the endophyte of *Myrica.*

The controversial views of earlier writers were discussed and it was concluded that the nodule organisms are members of the Plasmodiophorales.

Selective Media for *Haemophilus pertussis* and *parapertussis*. By B. W. LACEY (*Westminster School of Medicine, London*)

Bordet Gengou medium, described by Mackie and McCartney, can be improved by increasing the sodium chloride concentration to 0·85 %, and the Difco proteose peptone to 1·3 %. Of 350 substances tested by a radial-streak method on this medium only one: 4:4-diamidino stilbene (stilbamidine) showed selective inhibition of the pharyngeal flora which frequently obscures the growth of *Haemophilus pertussis* on Bordet Gengou medium, containing 0·3 unit penicillin/ml., inoculated with pharyngeal swabs. The inclusion of 32 μg./ml. stilbamidine diisethionate (May and Baker 744) and 0·3 unit penicillin/ml. reduces the incidence of the growth of organisms other than *H. pertussis* to about half, but the results of its routine use, shown in Table 1, have been disappointing.

Table 1

Total cases positive: 107

	From per-nasal swab		From post-nasal swab		From one or both swabs	
Medium	Positive cases	No. of 3½ in. plates	Positive cases	No. of 3½ in. plates	Positive cases	No. of 3½ in. plates
Bordet with penicillin	76	1	69	1	95	2
Bordet with penicillin and stilbamidine	70	¼	65	¼	87	½
One or both media	84	1¼	84	1¼	107	2½

Of forty other diamidines kindly supplied by Dr Ewins, May and Baker Ltd., or Dr King, Medical Research Council, Mill Hill, none was more selective than stilbamidine on Bordet Gengou medium. With a semi-synthetic medium however, (containing, in addition to horse blood: starch, agar, glycerol, glutamate, alanine, cysteine, sodium, potassium, magnesium, chloride, fluoride, aconitate, fumarate, malonate and 0·3 unit penicillin g./ml.) 4 : 4-diamidino diphenylamine dihydrochloride (May and Baker 938) at 12μg./ml. is highly selective for *H. pertussis*. On this medium the incidence of growth of organisms, other than *H. pertussis* or *parapertussis*, in pharyngeal swab cultures, is reduced to 4 %. With most organisms from pharyngeal cultures on Bordet Gengou with 0·3 unit penicillin /ml. (20/20 neisseria, 12/12 staphylococci, 20/20 *H. influenzae*, 19/20 streptococci, 18/20 corynebacteria, 5/6 candida, 15/20 coliforms) the proportion growing is reduced to less than 10^{-3}, while none of thirty strains of *H. pertussis* showed a reduction of more than 50 % in number of colonies or any reduction in colony size.

The results of a controlled trial are shown in Table 2.

Table 2

Medium	Area	Positive diagnoses		
		From per nasal (naso-pharyngeal) swab	From post-nasal (oro-pharyngeal) swab	From one or both swabs
1. Bordet-Gengou with penicillin	Whole plate	35 = 57·5%	32 = 52·5%	43 = 70·5%
2. Semi-synthetic with penicillin and M. and B. 938	¼ plate	43 = 70·5%	43 = 70·5%	58 = 95%
1, 2. One or both	1¼ plates	44 = 72%	47 = 77%	61 = 100%

4 : 4-diamidino diphenylamine dihydrochloride (May and Baker 938) appears also to be of potential use for (*a*) selective culture of Malleomyces pseudomallei and some pathogenic fungi, streptomyces and nocardia, (*b*) inhibiting the growth of moulds in primary cultures of *M. tuberculosis*.

Observations on the Destruction of Bacteria by a Soil Amoeba.

By J. W. Czekalowski, D. E. Dolby, K. I. Johnstone, C. J. La Touche and J. W. McLeod (*Leeds University*)

Observations refer to a 'pure' culture of a limax amoeba obtained from soil. The amoeba would not grow independently of bacteria hence the pure culture was one obtained by implanting a single amoebic cyst on a surface culture of a susceptible bacterium. Such observations are well known to soil microbiologists but have not received the attention they deserve in text-books of bacteriology.

For systematic examination of the ability of the amoeba to attack pathogenic bacteria very striking results were obtained by growing the bacterium under investigation as an abundant surface culture for 24 or 48 hr. at 37°, and then inoculating material containing the amoebae or cysts at margin of the plate, and then keeping at room temperature.

It was found that the bacteria fell into the following categories:

(1) Those which favoured rapid advance of the amoeba so that the bacterial culture was obliterated over the whole surface of the plate in 3 or 4 days. Such were bacteria of the colon, typhoid, salmonella and dysentery groups.

(2) Those which permitted a slower growth which, however, continued till the whole plate was overrun, such were *H. influenzae*, *Staph. aureus*, *Vibrio cholerae* and *pasteurellae*.

(3) Those which allowed only a slow advance of the amoeba terminating after 3–8 days before the plate was overrun. Such were *haemolytic streptococci*, *meningococci*, *brucella*, *pneumococci*.

(4) Those which did not permit of amoebic growth, as *C. diphtheriae*, *Vibrio metchnikoffi* and some anthracoids.

(5) Those which gave irregular results—the anaerobes *Ps. pyocyanea* and *B. subtilis*.

Under anaerobic conditions susceptible bacteria were not attacked.

An interesting phenomenon was that subculture on 'chocolate' from portions of a coli culture which had been overgrown by the amoebae yielded on subculture very scanty growths of isolated colonies of *B. coli* and that some days later these were converted to almost pure colonies of amoeba which had a violet or lilac colour and were frequently umbilicated.

The Effect of Temperature on the Nutrition of *Pasteurella pestis*. By Errol D. Spurr and G. M. Hills (*Microbiological Research Station, Porton*)

Rao (1939) and Doudoroff (1943), working with large inocula at 28°, found *Pasteurella pestis*, nutritionally less exacting than did Berkman (1942) and Herbert (1949) working at 37°. Since these differences might have been due to strain or inoculum size, we have re-examined the problem. Using viable counts as a quantitative measure of the growth of three virulent and three avirulent strains, we have confirmed the finding that phenylalanine is essential, but growth at 28° is delayed unless this amino-acid is supplemented by valine, isoleucine, cysteine, methionine and haemin. At 36° rapid growth occurred only in the presence of additional nutrients such as a mixture of alanine, leucine, serine, threonine, biotin and pantothenate. The four amino-acids of this mixture showed mutually inhibitory relationships, but omission of alanine or leucine merely delayed growth without reducing the peak counts. Biotin and pantothenate were replaceable by a mixture of glycine, norleucine, aspartate, glutamate, lysine, arginine, histidine, proline, hydroxyproline, tyrosine and tryptophan. Control of temperature was critical and growth was not observed consistently at 37·0 ± 0·5°.

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Some Quantitative Aspects of Drug Resistance.

By A. Mayr-Harting (*Department of Preventive Medicine, Bristol University*)

The Diffusion of Antiseptics through Agar Gels. II. The Effect of Temperature on the Assay of Streptomycin. III. The Temperature of Agar During the Early Hours of Incubation of Agar Plates and its Importance in Assays.

By K. E. Cooper, W. A. Gillespie and A. H. Linton (*Bristol University*)

The Interaction of Streptomycin and *Escherichia coli*.

By Kenneth McQuillen (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

Hotchkiss (1948), discussing the investigation of antibacterial action, mentions first 'the physical-chemical process by which the agent attains a toxic concentration upon or within the affected cell'. Gros & Macheboeuf (1949) reported that streptomycin agglutinated a large number of strains of bacteria but that those possessing the somatic 'O' antigen (Smooth strains of the coli/salmonella group) were not agglutinated. These Smooth strains were also notably more resistant to the action of the antibiotic than the corresponding Rough strains. Several workers have shown that streptomycin combines with nucleic acids, reacting as a multivalent base.

The interaction of streptomycin and a Rough strain of *Escherichia coli* has been analysed by the method of micro-electrophoresis. The antibiotic combines with surface groups of the organisms decreasing their net negative charge and resulting in agglutination. Combination and agglutination are readily reversed by washing the cells with M/10-NaCl or resuspending them in fresh broth. Growth of *Esch. coli* in the presence of 100 units/ml. streptomycin yields cells which, after washing, have normal mobilities although considerable inhibition of growth has occurred. When streptomycin-treated and untreated cells are mixed, there is an equal distribution of the drug amongst all the cells. The combination of streptomycin with surface groups of *Esch. coli* can be represented as an adsorption isotherm.

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The Inhibition of Glutamate Accumulation by Aspartate and Cysteine in *Staphylococcus aureus*.

By E. F. Gale and M. B. van Halteren (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

If washed suspensions of *Staphylococcus aureus* are incubated in the presence of glutamic acid and glucose, glutamic acid passes into the cells and accumulates therein in the free state. The rate of accumulation is decreased by the presence, in the external medium, of certain other amino-acids such as aspartic acid or cysteine. Aspartic acid competitively inhibits the processes involved in the internal accumulation of glutamic acid; the internal accumulation of aspartic acid being, in turn, inhibited by the presence of glutamic acid. No peptide formation occurs during incubation of the cells under these conditions. Cysteine decreases the rate of accumulation of free glutamic acid within the cells but the rate of disappearance of free glutamic acid from the external medium is slightly increased; combined glutamate is found to accumulate in the medium at a rate corresponding to the decrease in the accumulation of the free amino-acid within the cells. Chromatographic analysis shows the appearance of a peptide in the medium. The peptide has been eluted from the paper and hydrolysed; it contains glutamic acid and cysteine only.

Improvements in Experimental Fermenters. By M. Lumb (*Boots Pure Drug Co. Ltd., Nottingham*)

An experimental set of forty-eight fermenters is described, suitable for statistically designed experiments of submerged culture fermentations.

The fermenters are arranged in four frames, each holding four units of three vessels. The units are seated in thermostatically controlled water baths.

The vessels are made of Pyrex glass, rounded at the bottom and carry a flange at the top by which they are held erect in metal stands. The Pyrex vessels have an overall length of 20 in. and an internal diameter of 6 in. Total capacity is 9 l. and operating volume 3–5 l. Each vessel is sealed at the top by a gasket inset into a stainless steel cover. This carries a centre gland and stirrer, a thermometer pocket, a sample line, an inlet air pipe and an exit air line. All fittings in contact with the liquor are made of stainless steel.

The stirrer shaft glands are of a double purpose roller bearing type with upper and lower oil seals. The stirrer shafts terminate at the bottom in adjustable and replaceable link stirrers and above in flanges, designed to take flexible couplings that connect the vessels with mitre bevel gears carried on horizontal driving shafts. Gear ratios are available for all speeds within the range 200–1000 r.p.m.

Steam-jacketed master air filters are attached to the frames and smaller subsidiary air filters to the vessels on both air inlet and outlet lines. Special valve and air line arrangements allow the sample lines to be cleared of all old liquor prior to sampling.

The vessels are steam sterilized as separate units and aseptic attachment of the vessels to the inlet and outlet air lines is made in situ on the frames by rubber hose connexions.

DEMONSTRATIONS

Lilian E. Hawker and J. Fraymouth (*Botany Department, Bristol University*). 'The morphology and life histories of the organisms inhabiting the root nodules of certain non-leguminous plants.'

Lilian E. Hawker (*Botany Department, Bristol University*). 'Hypogaeous fungi.'

B. W. Lacey (*Westminster School of Medicine, London*). 'Selective media for *Haemophilus pertussis* and *parapertussis*.'

W. A. Gillespie (*Bristol Royal Infirmary*). 'A "biochemical mutant" of *Bact. coli* causing urinary tract infection.'

Prof. J. E. Harris (*Bristol University*). 'The "de Fonbrune" micromanipulator.

V. O. Nicholls (*Bristol University*). 'The morphology and pathogenicity of some root-rotting species of Fusarium.'

B. E. J. Wheeler (*Bristol University*). 'Experiments in nitrification.'

B. F. Folkes (*Bristol University*). 'Microbiological assay of amino-acids.'

[The Editors of the Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from authors.]*

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Twelfth General Meeting at the Royal Society of Medicine, London, on Monday and Tuesday, 16 *and* 17 *April* 1951. *The following communications were made:*

COMMUNICATIONS

SYMPOSIUM ON TRACE METALS AND MICROBIOLOGY

Chairman: SIR PAUL FILDES

The Affinity of Essential Cellular Constituents (Amino-acids, Pteridines, Purines and Riboflavine) for the Ions of Heavy Metals. BY A. ALBERT (*Department of Medical Chemistry, Australian National University*)

Metal Deficiency and Iron Catalysts in Bacteria. BY A. TISSIERES (*Molteno Institute, University of Cambridge*)

Metal Enzymes and Metal-Activated Enzymes. BY D. HERBERT (*National Institute for Medical Research, London*)

The Role of Iron and Manganese in so-called Iron Organisms. BY E. G. PRINGSHEIM (*Botany School, University of Cambridge*)

Trace Element Nutrition of Fungi with Special Reference to *Aspergillus niger*. BY D. J. D. NICHOLAS (*Long Ashton Research Station*)

Trace Elements and Antibiotic Fermentations. BY G. N. ROLINSON (*Bacteriology Department, Boots Pure Drug Company Ltd., Nottingham*)

Trace Metals and Toxins. BY W. E. VAN HEYNINGEN (*School of Pathology, University of Oxford*)

Antimicrobial Activity of Metals. BY A. J. WOIWOD (*Wellcome Research Laboratories, Beckenham*)

Summing up: B. C. J. G. KNIGHT

DEMONSTRATION

H. SANDON (*Gordon Memorial College, Khartoum*). 'Opalinids from Nile fish'

ORIGINAL PAPERS

Opalinids from Nile Fish. By H. Sandon (*Gordon Memorial College, Khartoum*)

Examinations of the gut contents of about 600 fish from Khartoum and the White Nile showed that a ciliate fauna consisting of Opalinids, *Balantidium* and *Nyctotherus* is characteristic of nine of the species. These nine species all belonged to the Characinoids and Siluroids, but within these groups there was little relation between the systematic position of the hosts and their susceptibility to this infection. The infected fish included mud-eaters (*Citharinus* spp.), vegetarians (*Distichodus rostratus*), predators (*Schilbe mystus, Eutropius niloticus*), zooplankton-eaters (*Synodontis batensoda, S. membranaceus*) and omnivorous scavengers (*Synodontis* spp.). The lack of relation between either systematic relations or ecological characters and ciliate infection is especially noticeable in the Schilbeidae represented by the three species, *Eutropius niloticus, Schilbe mystus* and *S. uranoscopus*, which resemble one another very closely in appearance, size, feeding and other habits and distribution. Yet, whereas specimens of the first two species are usually very heavily infected, the fifty-nine specimens examined of the last have all been completely free from ciliates.

A striking fact concerning the infections is the invariable association of the Opalinids with the heterotrichous ciliates. No fish species has been found in the course of this work subject to infection by the one group which has not also been infected by the other. Three genera of Opalinids are represented, *Protoopalina, Cepedea* and *Zelleriella*, of which the last is a characteristically South American type only once previously recorded from Africa. The species have not yet been determined owing to the very unsatisfactory state of the systematics of these organisms. As an example of the obscurities in the definitions of species, it was pointed out that the numbers of ciliary rows and the distances between them are commonly given as diagnostic characters, but a detailed study of the ciliary system in *Protoopalina* shows that incomplete rows are sufficiently common to make such data completely unsatisfactory. The *Zelleriella* is notable for an unusual mode of division as a result of which 4- and occasionally 8-nucleate individuals occur.

Unusual Behaviour of a Lysogenic *Bacillus* Strain. By Elinor McCloy (*London School of Hygiene and Tropical Medicine*)

Cultures of the *Bacillus* strain W, allowed to age in large volumes of broth, give rise on plating to growth which has eroded patches in it. These patches are due to the action of a bacteriophage, and filtrates of such cultures contain this phage, phage α, which attacks and can be propagated on strain W itself, and on strains of *B. anthracis*. Filtrates of young cultures of strain W contain a phage, phage β, which attacks strains of *B. anthracis*, but not strain W. Since the two phages are serologically identical, and resemble each other in all characters except ability to attack strain W, it is postulated that the intracellular precursor of each is the same, and is normally the precursor of the β form, phage α arising as a rare mutant. The properties of lysogenic strains which protect them from attack by those phages adsorbed from the exterior whose intracellular precursors they carry, and which protect strain W from externally applied phage β, do not protect strain W from the variant phage α.

Conditions prevailing in ageing cultures appear to be necessary for manifestation of phage α, for phage α has been obtained only from such cultures. No phage particles capable of attacking strain W have been found in preparations of phage β propagated to high titre on heterologous strains.

β-resistant, α-sensitive variants of *B. anthracis* strains have been obtained through the action of phage β. These constitute artificially produced models of strain W for they are lysogenic, liberating phage β in young cultures, and a culture of one of them allowed to age in a large volume of broth yielded eroded growth on plating, and a phage which is able to attack the β-resistant variants from which it was derived.

Some Aspects of the Metabolism of a New Group of Iron-Oxidizing Micro-organisms in Soil. By H. Gleen (*Long Ashton Research Station, Bristol*)

A soil-perfusion method was used to investigate the role played by soil micro-organisms in the fixation of ferrous iron in soil. Divalent iron perfused through soil was chemically precipitated at pH 5·0 and over, though remained chemically stable at acid pH 3·0–3·5. The growth, at acid pH 3·0, of a specific iron-oxidizing microflora on soil, with their eventual saturation of the soil crumb surface forming an 'enriched' soil was shown; this soil can be regarded as an enzyme system specific for iron activation. 0·01 % sodium azide inhibits this biological oxidation completely. The growth of these micro-organisms appears to be dependent on the optimum diffusion of oxygen in the film of fluid coating the soil particles, and the presence of a growth factor in the soil. They do not proliferate in the perfusate on sand or pumice.

The incidence of these micro-organisms in some soils is high, and low concentrations of divalent iron are metabolized without a lag phase though higher iron concentrations appear to extend this lag. There is no influence of these high iron concentrations on iron metabolism once the soil is enriched.

The influence of sulphonamide, 2:4-dinitro-*o*-phenol, *p*-amino-benzoate, ammonium and nitrate, on growth and metabolism of these iron organisms in soil was shown.

It appears that these iron-oxidizing micro-organisms can activate thiosulphate, oxidizing it to tetrathionate.

Oxygen uptake studies of enriched soils demonstrate that the rate of O_2 uptake is dependent on the divalent iron concentration, and is inhibited by sodium azide, iodoacetic acid and mercuric chloride. It was shown that $S_2O_3^{--}$ and Fe^{++} are activated by the same enzyme system, and that both the rate of O_2 uptake due to Fe^{++} and $S_2O_3^{--}$ was increased in the presence of phosphate.

Variation and Inheritance of Penicillin Sensitivity in Single-Cell Cultures of *Staphylococcus aureus*. By W. Howard Hughes (*Wright-Fleming Institute, London*)

A study of the distribution of penicillin resistance in a population of *Staphylococcus aureus* was made, using subcultures from single cells picked with a micromanipulator.

The original strain was obtained from a previously untreated breast abscess, a single colony being selected from the pure growth obtained.

Two single-cell cultures were made from this and compared with it for sensitivity. Using steps of $\frac{1}{400}$ of a unit in plates it was found that a constant difference of that amount could be demonstrated between the two daughter strains: (I) being identical with the parent, but (II) being $\frac{1}{400}$ unit/ml. more resistant. This difference is still present after over a year in artificial culture.

The end-point using small implants, up to 300 organisms, was very sharp; nevertheless, an increase in implant gave survivors at all levels up to four times the original concentration. The increase of implant necessary to give a 10% increase in resistance being about tenfold.

From these survivors further single-cell cultures were made to give other generations. These gave similar patterns and similar isolated survivors from heavier implants.

Having obtained these generations they were titrated, and it was found that the distribution of resistant forms followed a consistent pattern. If the survivors are expressed in numbers per million implant and the log of this is plotted against the log of the dose of penicillin, the result is approximately a straight line and parallel with that of the other generations; the pattern is of a distributed population.

Back variation to sensitivity is also found. No method for selecting for it, or for expressing it quantitatively, is available at present.

The Segregation of Streptomycin-Resistant Variants of *Mycobacterium tuberculosis* into Groups with Characteristic Levels of Resistance to the Antibiotic. By D. A. Mitchison (*Postgraduate Medical School, London*)

Bacterial Reduction of Dehydroascorbic Acid. By B. P. Eddy, M. Ingram and L. W. Mapson (*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research*)

Though it has been known for some time that bacteria can reduce dehydroascorbic acid (DHA), there has hitherto been no clear indication of which organisms effect the change, nor of the reaction mechanism. Examination of a variety of species has shown that only staphylococci and members of the Enterobacteriaceae bring about the reduction. With intact cells, various substrates served as H-donators; but cell-free dehydrogenases were ineffective.

Further, methylene blue was reduced more quickly than DHA; and if two H-donating substrates were present, their contributions to the total rate of DHA-reduction were not additive. Hence it appears that some factor (possibly a carrier) is involved, additional to the H-donating system.

Besides possessing the ability to reduce DHA, the staphylococci and Enterobacteriaceae are peculiar in containing cytochrome b_1. Spectroscopic observations have indicated that in reducing DHA the cytochrome b_1 is oxidized (though incompletely). We therefore suppose that cytochrome b_1 may be the H-carrier.

We have, however, found bacteria possessing cytochrome b_1 which cannot reduce DHA, so that an additional factor must be postulated. This may be a DHA reductase, because leuco-Nile-blue is re-oxidized by DHA more strongly in the presence of cells of a DHA-reducing strain than those of a non-reducer.

Some Observations on the Effect of Antibiotics on a Strain of *Escherichia coli*. By C. H. Smith (*Institute of Ophthalmology, London*)

Penicillin, aureomycin and chloromycetin were added to cultures of the *Esch. coli* strain B, used for propagation of the T series of bacteriophages. When the antibiotic was added to cultures in the log phase of growth penicillin and aureomycin caused a sudden drop in viable count. The rate of fall was proportional to the concentration of antibiotic added. Chloromycetin did not produce this fall, its action being essentially bacteriostatic.

When concentrations of penicillin and chloromycetin near to the inhibitory level were added there was a period of 30 min. in which the rate of growth persisted in the log phase before the antibiotic exerted its effect. This was not found with aureomycin.

Exposure of the organism to action of the antibiotic for 15 min. periods showed that those organisms which survived penicillin multiplied at the same rate as the control organism, whereas in those who survived chloromycetin and aureomycin the generation time was increased, with chloromycetin by $1\frac{1}{2}$ times and aureomycin by 3 times that found in the control.

Studies on the Nitrifying Organisms. By H. Lees (*Department of Biological Chemistry, Aberdeen University*)

The method of culturing the soil-nitrifying organisms in highly aerated media (Lees, 1951) will be described in detail. The nitrite-forming organisms so isolated formed nitrite from ammonium salts, but not from any of the common amino-acids nor from urea. All the common amino-acids, including methionine, were tested for their ability to inhibit nitrite formation at a concentration of 2×10^{-3} M amino-acid in 1×10^{-3} M/$(NH_4)_2SO_4$. No amino-acid, with the exceptions of histidine and possibly arginine, showed any inhibition. The report that methionine is toxic to nitrification in soil (Quastel & Scholefield, 1949) has been confirmed; possible reasons for the different behaviour of methionine in culture and in soil will be suggested. Sodium diethyldithiocarbamate, which is toxic to nitrification in soil (Lees, 1946, 1948) prevented nitrite formation in culture at a concentration of 10^{-5} M; potassium chlorate (5×10^{-4} M) which does not inhibit nitrite formation in soils (Lees & Quastel, 1945) was equally without effect in culture. Potassium penicillin showed a just detectable inhibition at 10 p.p.m.; the $CaCl_2$ salt of streptomycin was markedly inhibitory at a dilution of 0·01 p.p.m. The significance of these results in relation to nitrification in soil will be discussed.

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Inhibition by Serum of the Aerobic Growth of a *Shigella* Strain. By B. Stocker (*London School of Hygiene and Tropical Medicine*)

On some batches of nutrient agar, *Shigella flexneri* strain F 4 fails to give visible growth from small inocula incubated aerobically overnight; the addition to any nutrient agar of serum, or of some other undenatured proteins, makes it similarly inhibitory. Under anaerobic conditions there is no inhibition. The addition of haematin makes some, but not all, batches of nutrient agar non-inhibitory, but has no effect on inhibition by serum. Serum does not act directly on the bacteria, for the growth of cells separated from serum-agar by cellophane is also inhibited. The inhibition is not due to peroxide, since it is not reversed by catalase or MnO_2. Treatment of inhibitory agar with activated charcoal makes it non-inhibitory, presumably by removal of some noxious substance; but charcoal does not reverse inhibition by serum.

Of growth factors tested, only the reducing substances cysteine and ascorbic acid show a (feeble) inhibition-reversing activity; the inhibition is entirely reversed by ferrous or ferric salts, in concentrations of 10^{-5} to 10^{-4} M for inhibitory nutrient agar, and of 10^{-2} M for serum-agar. It is also reversed by a substance, 'S', produced by all *Shigella* strains tested (including F 4 itself), by some other genera of Gram-negative rods, and by some *Bacillus* species. This substance is filterable and dialysable; it is heat-stable and active in high dilutions.

It is hypothesized that 'S' is a normal metabolite of *Shigella* strains, and one that is synthesized with difficulty by isolated cells of strain F 4; that 'S' is a component of an enzyme system required for the intracellular neutralization of an inhibitor formed in the medium under aerobic conditions, the formation of the inhibitor being enhanced by serum, perhaps through its oxygen-carrying effect; and that the enzyme system can be replaced by high concentrations of iron, which may indicate that the enzyme incorporates an iron-porphyrin molecule.

Haematin as a Growth Factor for a Strict Anaerobe, *Fusiformis melaninogenicus*. By R. J. Evans (*London School of Hygiene and Tropical Medicine*)

Fusiformis melaninogenicus is a strict anaerobe which will grow only on media containing blood or blood derivatives. Experiments have shown that it will not grow on tryptic digest agar unless haematin is added; coenzyme I neither supports growth nor does it cause any increase of growth in the presence of optimum amounts of haematin. A relationship has been shown between the size of the inoculum and the amount of haematin required for growth; there is evidence that the organism is unable to synthesize haematin, and that it has an absolute requirement for this substance as a growth factor. Viable counts performed on media containing varying amounts of haematin show that the number of colonies developing from a given inoculum decreases as the concentration of haematin in the medium falls; a hypothesis is presented to explain this phenomenon. Growth of the organism is completely inhibited by low concentrations of cyanide or azide added to a medium containing sufficient haematin to support full growth in their absence.

Haematin is therefore an essential growth factor for the strict anaerobe, *F. melaninogenicus*, and is probably required for incorporation into an iron-porphyrin enzyme.

The Rate of Tetrathionase Formation. By R. Knox (*Guy's Hospital Medical School, London*)

Washed suspensions of tetrathionate-reducing organisms such as *Salmonella paratyphi* B, or coliform '1433', show two main types of adaptation curve: (1) with tetrathionate alone adaptation is very slow, and a linear relation is obtained between the amount of substrate removed and the *square* of time, indicating that enzyme is being formed at uniform rate; (2) with tetrathionate and a rich energy source such as mannitol or glucose, adaptation occurs slowly at first, then at a rapidly increasing rate until adaptation is complete. Although when enzyme concentration is plotted against time an S-shaped curve is obtained of the self-limiting autocatalytic type (Spiegelman), an equally good fit is given by plotting the amount of tetrathionate reduced against the *cube* of time, suggesting that enzyme is being produced with uniform *acceleration*.

It has previously been shown that washed cells incubated first with mannitol and phosphate show a greatly reduced adaptation time when tetrathionate is added. With such cells a quadratic, not a cubic equation, is obtained, suggesting that during pretreatment in the absence of the substrate precursor or at least some factor controlling enzyme formation has been built up, so that on subsequent addition of the substrate enzyme formation occurs rapidly and at uniform rate.

These facts suggest that in full enzymic adaptation, two processes are involved: (1) conversion of precursor into enzyme, specifically induced by the substrate; and (2) formation of more precursor, less specific and dependent on a good source of energy. If we assume that the rate of conversion of precursor into enzyme is itself proportional to the concentration of precursor, then the second process becomes rate-determining for the first.

The experimental data obtained with tetrathionase lead then to a concept of enzyme formation which embraces in modified forms both the mass action theory of Yudkin with its emphasis on the part played by the specific substrate and the 'building stone' theory of Jacoby with its emphasis on non-specific factors.

The Formation of Bacterial Urease. By R. Knox (*Guy's Hospital Medical School, London*)

Washed suspensions of *Proteus* prepared from cultures grown on nutrient agar show a basal urease activity which is not increased by incubation of the washed cells with urea, but is increased 2- to 3-fold by incubation with glucose and phosphate, and 5- to 20-fold by incubation with glucose, phosphate and urea at 37°. As with tetrathionase, two factors seem to be involved—specific and non-specific. Whereas with tetrathionase previous incubation of washed cells with glucose results not in an increase of enzyme but in an increased rate of enzyme formation when specific substrate is added, with urease there is actually an increase of enzyme itself during this preliminary incubation in the absence of specific substrate. The formation of urease in washed suspensions, though less dependent on the presence of the specific substrate, resembles the formation of tetrathionase: (1) in its dependence upon a source of energy such as fermentable carbohydrate; (2) in its sensitivity to temperatures such as 45–48° at which the enzyme itself is fully active; and (3) in its susceptibility to an inhibitor of synthetic processes such as azide which has little inhibitory effect on the enzyme itself.

Hydrodynamic Aspects of Bacterial Locomotion. By D. Herbert (*National Institute for Medical Research, London*)

Some problems relating to bacterial motility are discussed from the standpoint of hydrodynamics. Analogies between the locomotion of bacteria and of larger aquatic animals are misleading, since the factors affecting fluid flow around large bodies moving fast and minute bodies moving slowly are qualitatively as well as quantitatively different. The Reynolds numbers characterizing the locomotion of aquatic animals range from 10^4 to 10^8, while those characterizing bacterial motion range from 10^{-4} to 10^{-5}. Moving bacteria are therefore subjected to a purely viscous drag, inertial forces being negligible. The fluid flow around moving bacteria must be laminar under all conditions, and considerations of shape are relatively unimportant.

The hydrodynamic drag on a moving bacterium can be calculated from Stokes's equation, and is minute. The power output required for propulsion could be achieved by combustion of approx. 2 glucose molecules per bacterium per sec., which is less than 0·1 % of the total metabolism.

The tangential shearing force on a moving bacterium is also minute, and inadequate to draw out a surface 'slime layer' into 'tails' as postulated by Pijper. The apparent 'gyrating undulating motion' described by this author can probably be attributed to irregular 'firing' of flagella, combined with a 'wobble' due to translational and rotational Brownian movements.

The hypothesis that flagella are true locomotory organs, consisting of a primitive type of muscle, does not appear to contradict any known facts. If their power-to-weight ratio is the same as that of muscle, the power available should be more than adequate for propulsion.

Antigenic Modulation of *Haemophilus pertussis*. By B. W. Lacey (*Westminster Medical School, London*)

H. pertussis grows well on a solid medium containing, in addition to 33 % horse blood, agar, glycerol, starch, cysteine, glutamate, alanine, sodium chloride and tap water. Substitution of the sodium chloride by an equivalent amount of other salts induces remarkable changes. With magnesium sulphate growth is more rapid, butyrous, more emulsifiable, more stable in suspension, never adherent to medium, less viable, less sensitive to 4:4-diamidino-diphenylamine (M & B 938), develops no haemagglutinin and is not agglutinated by rabbit serum against sodium chloride growth of either *H. pertussis* or *H. parapertussis*. Rabbit serum against magnesium sulphate growth does not agglutinate *H. parapertussis* and agglutinates sodium chloride growth of *H. pertussis* to a lower titre than magnesium sulphate growth. It seems probable, from a preliminary experiment, that magnesium sulphate growth compared with sodium chloride growth is also less virulent for mice intranasally, and is less protective against intranasal challenge. All of one hundred strains of *H. pertussis* and all of one hundred colonies of one strain have shown these antigenic changes. Three strains of *H. parapertussis* and one strain of *H. bronchiseptica* tested have not.

The changes are not reproduced by suspension in salt solutions, nor do they occur during twenty days at 5°. They are completely reversible: one strain has been taken through six cycles. At 36° equilibrium is reached in 15–72 hr. Viable counts on both media from either are the same. Thus the change appears to be a phenotypic modification (or modulation) requiring five to twenty subcultures to become fully manifest or reversed.

Analysis of the modulatory effect of one hundred salts or mixtures on the slide agglutinability of both *H. pertussis* and *H. parapertussis* by twelve unabsorbed and numerous absorbed rabbit sera indicates that, within Phase I, *H. pertussis* has four major evocable agglutinogens (1, 2, 4 and 5), and *H. parapertussis* two permanent major agglutinogens (2 and 3). Convalescent pertussis sera and commercial pertussis vaccines vary both qualitatively and quantitatively.